REMARKS

Applicants acknowledge receipt of the Communication mailed March 23, 2004.

Applicants thank Examiner Parkin for extending the courtesy of a productive telephonic interview to Applicants' representative on August 17, 2004. Matters discussed during the interview included evidence for unexpectedly good results achieved using a primer that included the target-complementary sequence of SEQ ID NO:10 (i.e., the promoter-primer of SEQ ID NO:15), the structural relationship between SEQ ID Nos:10 and 15, and the alignment of the target-complementary sequences identified in Table 2. Also discussed were the optional nature of 5' promoter sequences that can be appended to primers, and language considered appropriate for defining a "kit."

Claims 59-61, 64 and 73 have been canceled, and Claims 55, 58, 62, 66, 68-69, 72, 75 and 78 have been amended by this response.

The Title of the Application is amended herein to reflect more appropriately the presently claimed invention.

The amendments to the claims are supported by the original disclosure. Support for the amendment of Claim 55 related to a "5' promoter sequence non-complementary to the HIV-2 nucleic acid" can be found under the definition appearing in the Specification on page 11 at lines 8-12, and in the paragraph bridging pages 16-17. Regarding the recitation of a sequence identifier in Claim 55, Applicants point out that Table 2 presents HIV-2 complementary primer sequences and corresponding T7 promoter-primer sequences, and that the HIV-2 complementary portion of the T7 promoter-primer of SEQ ID NO:15 is SEQ ID NO:10. Disclosure concerning unexpectedly good results that can be achieved with the presently claimed combinations is discussed below in connection with the § 103(a) rejection. Claims 58 and 62 have been amended to conform with the limitation in Claim 55 which increases the lower limit on the length of the first amplification oligonucleotide. Claim 62 has been further amended to clarify the presence of an upstream promoter sequence in the first amplification oligonucleotide. Claims 66 and 69 have been narrowed to specify a single promoter-primer sequence. Claim 68 has been amended to

clarify the presence of an upstream promoter sequence in the first amplification oligonucleotide. A typographical error that resulted in two claims numbered "71" has been corrected by renumbering the second instance as Claim "72." Renumbered Claim 72 has been amended narrow the length description of the first amplification oligonucleotide in conformance with the amendment of Claim 55. Claim 75 has been amended to depend from Claim 72 instead of from canceled Claim 73. Finally, Claim 78 has been amended to reduce the number of recited first amplification oligonucleotides in conformance with the amendment of Claim 55.

A Substitute Sequence Listing in paper and computer-readable versions is enclosed.

Claims 55-58, 62-63, 65-72 and 74-90 will be pending after entry of this Amendment.

No new matter is being added by the amendments made herein.

Entry of this Amendment is respectfully requested.

Compliance with 37 C.F.R. § 1.821-1.825

The Examiner has requested a duplicate copy of the Sequence Listing because the original of page 4 is missing. A substitute copy of the Sequence Listing, in both paper and computer-readable versions is enclosed. This satisfies the Examiner's request for compliance with the Sequence Listing requirements.

Compliance with 37 C.F.R. § 1.98

The Examiner has indicated that the IDS filed February 12, 2003, did not comply with 37 CFR § 1.98(a)(2), apparently because copies of the cited references were not paired up with the file. Copies of the 8 references cited in the subject IDS, together with a copy of the IDS mailed February 11, 2003 (marked "COPY"), are provided herewith. Applicants request that the Examiner consider the enclosed references and return to Applicants an initialed copy of the SB/08B form.

<u>The Rejection Under 35 U.S.C. § 112, Second Paragraph</u>

Claims 55-90 have been rejected under 35 USC § 112, second paragraph as being indefinite because structural components of the claimed kit are not clearly set forth. More specifically, the rejection indicates that the structural components of the kit, such as containers/vials and instructions/directions for using the kit, are not recited in the claims.

Responsive to the rejection, Claim 55 has been amended to recite a "packaged combination" of components "in one or more containers." The term "kit" is used in the instant application to describe packaged combinations that include amplification oligonucleotides, and perhaps probes as well, that can be used for detecting HIV-2 nucleic acids (see the paragraph bridging pages 31-32 of the Specification). One of ordinary skill in the art would understand that the instant claims embrace kits having first and second amplification oligonucleotides in one or more containers, which, in turn, are contained in the same bundle of things packed or wrapped together (i.e., a "package"). Accordingly, since the scope of the claims would be clear to one of ordinary skill in the art in view of the Specification, and so Applicants request withdrawal of the rejection under § 112, second paragraph.

<u>The Rejection Under 35 U.S.C. § 103(a)</u>

Claims 55-90 have been rejected under 35 USC § 103(a) as being *prima facie* obvious in light of the combined teaching of Pieniazek et al., Myers et al., Nelson et al., and Sorge et al. The rejection suggests that, because Pieniazek et al., disclose a single pair of primers and one probe for detecting HIV-2 nucleic acids, because Myers et al., disclose the polynucleotide sequences of the LTRs for different HIV-2 isolates, because Nelson et al., disclose chemical modifications of probes and primers to enhance activity, and because Sorge et al., disclose various primers and probes (including T7-based primers), it would have been routine for an ordinary skilled artisan to have created the instantly recited amplification oligonucleotides and probes, and to have packaged

them into a kit for detecting HIV-2 nucleic acids. Simply stated, the rejection is founded on the premise that knowledge of the structure of the HIV-2 LTR, combined with routine experience in the preparation of primers renders obvious the primer structures recited in the claims.

When examining the obviousness of a new compound that is structurally related to prior art compounds, all of the properties of the relevant molecules must be considered. The Court of Customs and Patent Appeals in *In re Papesch*, 137 U.S.P.Q. 43 (C.C.P.A. 1963), stated that:

> From the standpoint of patent law, a compound and all of its properties are inseparable; they are one and the same thing.... And the patentability of the thing does not depend on the similarity of its formula to that of another compound but of the similarity of the former compound to the latter. There is no basis in law for ignoring any property in making such a comparison.

Under the *Papesch* doctrine, evidence of unobvious or unexpected advantageous properties may rebut a *prima facie* case of obviousness based on structural similarities. (*In re Chupp*, 816 F.2d 643, 2 USPQ2d 1437 (Fed. Cir. 1987)) Since a polynucleotide is a compound, albeit a complex one, and since all properties of a compound are material to patentability, it follows that all properties characteristic of the instantly recited polynucleotide components of the claimed kit are material to patentability and must properly be considered.

The Specification presents results from a series of comparative experiments using different combinations of opposed primers to amplify a model HIV-2 template nucleic acid, and specifically identifies one primer as having particularly desirable properties when used in combination with any of several second-strand primers. Indeed, Table 4 summarizes results from a procedure using three different first-strand primers (SEQ ID Nos:15-17) in paired combination with three different second strand primers (SEQ ID Nos:2-3 and 7). The results indicated that the primer having SEQ ID NO:15 gave uniformly high levels of amplification (as judged by "Avg. RLU" values exceeding 10 million) with excellent reproducibility (as judged by advantageously low "%CV" values) in

reactions that included 5,000 copies of the HIV-2 template. The Specification emphasizes the highly desirable characteristics of this primer on page 35 at line 17, where it is stated:

> Although all of the promoter-primers in the procedure gave easily detectable amplification signals, the promoter-primer identified as SEQ ID NO:15 advantageously gave good results when used in combination with each of the non-T7 primers that was [*sic*] tested. Notably, amplification reactions that included the promoter-primer having the sequence of SEQ ID NO:15 uniformly were associated with low %CV values, thereby indicating a high degree of reproducibility and particular robustness of amplification reactions that included this primer.

The superior amplification capacity of primer combinations that included SEQ ID NO:15 is reiterated in Tables 5 and 6, which provide a comparison with a first-strand primer identified by SEQ ID NO:17. Signal-to-noise ("S/N") ratios (calculated as Avg. RLU ÷ Neg. control) for reactions conducted using only 100 copies of the HIV-2 template ranged from about 113-118 when the primer of SEQ ID NO:15 was used in combination with any of three second-strand primers, but ranged from only about 1-10 when the primer of SEQ ID NO:17 was used instead. Notably, SEQ ID NO:15 and SEQ ID NO:17 differ by two contiguous insertion/deletions and one purine substitution over the lengths of the primer sequences. The results in Table 7 again confirmed that amplification reactions conducted using the primer of SEQ ID NO:15 in combination with each of three new second-strand primers gave very high levels of amplification (Avg. RLU signals uniformly exceeded 10 million) with advantageously low %CV values. Calculated S/N values for reactions conducted using the primer of SEQ ID NO:15 in combination with the second-strand primers were 114 (for SEQ ID NO:4), 183 (for SEQ ID NO:5) and 1189 (for SEQ ID NO:6) for reactions that included only 100 copies of the HIV-2 template. Taken together, these results show that the combination of a first-strand primer consisting of SEQ ID NO:15, together with any of several second-strand primers which are embraced by the language of the instant claims yield a robust amplification reaction capable of detecting low levels of the HIV-2 target with high S/N values in a highly reproducible manner.

To aid in better understanding how the relevant "first amplification oligonucleotides" disclosed in the Specification relate to each other, two alignments are presented below. Each of the alignments includes instant SEQ ID NO:9, which is fully complementary to the HIV-2 consensus sequence presented by Myers et al., on the line terminating with "816" (page I-B-7). Alignment 1 shows the relationship among the HIV-2 LTR-complementary primer sequences of SEQ ID Nos:10-14, and identifies the corresponding promoter-primer sequences. The information in Alignment 1 is found in the paragraph bridging pages 21-22, and in Table 2 of the instant Specification.

Alignment 1

```
                                            Target-             Promoter-primer
                                            complementary       containing the
                                            sequence            sequence
5'-GTCCCTGTTCGGGCGCCAACCTGCTAGGGATTTT-3'   SEQ ID NO:9

         5'-CGGGCGCCAACCTGCTAGGGATTTT-3'   SEQ ID NO:10        SEQ ID NO:15
5'-GTCCCTGTTCGGGCGCCA-3'                   SEQ ID NO:11        SEQ ID NO:16
         5'-CGGGCGCCA--CTGCTAGAGATTTT-3'   SEQ ID NO:12        SEQ ID NO:17
         5'-CGGGCGCCA-CCTGCTAGGGATTTT-3'   SEQ ID NO:13        SEQ ID NO:18
  5'-CCCTGTTCGGGCGCCAACCTGCTAG-3'          SEQ ID NO:14        SEQ ID NO:19
```

Alignment 2 illustrates the relationship between the HIV-2 LTR-complementary primer of SEQ ID NO:10 and the promoter-primer of SEQ ID NO:15 which contains the sequence of SEQ ID NO:10 in addition to an upstream T7 promoter sequence. The T7 promoter sequence, which is highlighted by underlining in Alignment 2, is not complementary to the HIV-2 nucleic acid.

Alignment 2

```
                     5'-GTCCCTGTTCGGGCGCCAACCTGCTAGGGATTTT-3'   SEQ ID NO:9
                              5'-CGGGCGCCAACCTGCTAGGGATTTT-3'   SEQ ID NO:10
5'-AATTTAATACGACTCACTATAGGGAGACGGGCGCCAACCTGCTAGGGATTTT-3'   SEQ ID NO:15
          (T7 promoter)
```

Despite the fact that the sequences of SEQ ID Nos:10 and 15, SEQ ID Nos:11 and 16, and SEQ ID Nos:14 and 19 are complementary to the consensus of Myers et al., evidence appearing in the Specification and summarized in the table below compels a conclusion that the

primer of SEQ ID NO:15, which contains the target-complementary sequence of SEQ ID NO:10, possesses unusually beneficial properties which distinguish it from other primers that were tested.

For the Examiner's convenience, the following table summarizes the results of amplification procedures conducted using only 100 copies of the HIV-2 template per reaction. The first two columns identify primer combinations used in each trial. The third column identifies the Table in the Specification which contains the information reproduced here. The fourth and fifth columns present the numerical values for hybridization signals measured for reactions that included or omitted, respectively, the HIV-2 template. The last column shows the signal/noise value calculated using data from the two previous columns.

1st-Strand Primer (SEQ ID NO)	2nd-Strand Primer (SEQ ID NO)	Data Source (Table No.)	Signal (Avg. RLU)	Noise (Neg. Control)	S/N
15	2	6	10,805,600	91,900	117
15	3	6	9,581,007	84,559	113
15	7	6	11,022,067	93,300	118
15	4	7	15,143,148	133,313	114
15	5	7	15,149,932	82,829	183
15	6	7	13,731,644	11,547	1189
16 (discontinued after 5k copy trial)	NA	NA	NA	NA	NA
17	2	6	1,710,494	497,960	3
17	3	6	129,235	115,172	1
17	7	6	11,022,067	93,300	9
18	6	8	2,800,000	7,367	380
19	6	8	2,800,000	36,207	77

The tabular summary shown above illustrates how combinations of first- and second-strand primers, where the first primer was the promoter-primer of SEQ ID NO:15, yielded unexpectedly good results when compared with other primer combinations. Notably, the Specification instructs in the paragraph bridging pages 16-17 that promoter-primers "can be modified by removal of, or synthesis without, a promoter sequence and still function as a primer." This is the basis for the recitation of SEQ ID NO:10 in Claim 55. As indicated above, the sequence of SEQ ID Nos:10 and 15 have identical HIV-2 LTR-complementary sequences, and differ only by the absence or presence of a 5' sequence which is non-complementary to the HIV-2 nucleic acid.

Since unexpected properties of exceptionally useful primer combinations must be considered in an obviousness-type analysis, since the first-strand primer identified by SEQ ID NO:15, which contains the HIV-2 complementary sequence of SEQ ID NO:10, evidenced superior activity when used in combination with numerous second-strand primers embraced by the instant claims, since nothing in the aggregated prior art would have provided the necessary guidance that an ordinary skilled artisan would require to arrive at the recited primer combinations, and since independent Claim 55 requires the presence of the HIV-2 complementary sequence of the surprisingly active primer identified by SEQ ID NO:15, it follows that the invention of Claim 55 cannot be considered *prima facie* obvious in view of the prior art. Since the remaining claims incorporate the limitations of non-obvious Claim 55 by virtue of their dependency thereon, whether direct or indirect, the dependent claims also are non-obvious. Notwithstanding the content of Nelson et al., and Sorge et al., nothing in either of these references could have supplemented the disclosures of Pieniazek et al., and Myers et al., in a manner that would have suggested how to create oligonucleotides having the unexpectedly good properties of the presently claimed combinations. Accordingly, the amended claims cannot be considered obvious under § 103(a), and so withdrawal of the rejection is respectfully requested.

VERIFICATION UNDER 37 C.F.R. §1.825(a) & (b)

All of the nucleotide sequences in the attached Sequence Listing were included in the application as filed. Pursuant to 37 C.F.R. §1.825(a), no new matter is being added herewith. As required under 37 C.F.R. §1.825(b), I hereby verify that the data on the enclosed disk and the paper copies of the Sequence Listing are identical.

Conclusion:

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding objections and rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the telephone number shown below.

Respectfully submitted,
GEN-PROBE INCORPORATED

Dated: Aug. 19, 2004

By: ______________________

Michael J. Gilly
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(858) 410-8657

H:\GP117\117-03.UT.ROA1.wpd
August 19, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	)	Group Art Unit: 1648
	)	
YANG *et al.*	)	Examiner: Parkin, J.
	)	
Serial No. 10/001,407	)	Atty. Docket No. GP117-03.UT
	)	
Filed: October 22, 2001	)	Confirmation No. 3070
	)	
For: COMPOSITIONS AND METHODS FOR DETECTING HUMAN IMMUNODEFICIENCY VIRUS 2 (HIV-2)	)	

COPY

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97(b)(3) & (e)(1)

Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants bring to the Examiner's attention the references listed on the enclosed form PTO/SB/08 (two copies) for consideration in connection with the examination of the above-identified application. A copy of each of the references is enclosed.

This Information Disclosure Statement is being submitted under 37 C.F.R. § 1.97(b)(3) before the mailing date of a first Office action on the merits.

Alternatively, if a first Office action on the merits was mailed on or before the certified mailing date indicated below, then, in accordance with 37 C.F.R. § 1.97(e)(1), the undersigned certifies that each item of information contained in this Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign

Certificate of Mailing

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date indicated below with sufficient postage as first class mail addressed to the Commissioner for Patents, Washington, D.C. 20231.

Date: February 11, 2003 By: [signature]

Michael J. Gilly, Reg. No. 42,579

application not more than three months prior to the filing of this Information Disclosure Statement. Accordingly, no fee is believed due in connection with this Information Disclosure Statement. If Applicants are mistaken, please charge the amount due to Deposit Account No. 07-0835 in the name of Gen-Probe Incorporated.

Applicants respectfully request that the Examiner indicate consideration of the cited references by returning a copy of the attached form PTO/SB/08 with the Examiner's initials or other appropriate marks.

Respectfully Submitted,

Date: February 11, 2003

By: ______________________
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Agent for Applicants

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Substitute for form 1449B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	10/001,407
Filing Date	October 22, 2001
First Named Inventor	YANG et al.
Group Art Unit	1648
Examiner Name	Parkin, J.
Attorney Docket Number	GP117-03.UT

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		BERKHOUT et al., "Secondary Structure of the HIV-2 Leader RNA Comprising the tRNA-Primer Binding Site", Nucleic Acids Res., Mar. 1993, 21(5):1171-1178	
		BERKHOUT et al., DATABASE GSN, Online!, Jun. 1993, retrieved from EBI Database accession no. X72325	
		BUSH et al., "Detection of Human Immunodeficiency Virus Type 1 RNA in Plasma Samples from High-Risk Pediatric Patients by Using the Self-Sustained Sequence Replication Reaction", J Clin Microbiol., Feb. 1992, 30(2):281-286	
		DE BAAR et al., "Design and Evaluation of a Human Immunodeficiency Virus Type 1 RNA Assay Using Nucleic Acid Sequence-Based Amplification Technology Able to Quantify Both Group M and O Viruses by Using the Long Terminal Repeat as Target" J Clin Microbiol., Jun. 1999, 37(6):1813-1818.	
		EIKEN CHEM CO LTD., DATABASE GSN, Online!, July 1998, retrieved from EBI Database accession no. AAV19509	
		KRAISELBURD, DATABASE GSN, Online!, Jun. 1996, retrieved from EBI Database accession no. AAT06604	
		PIENIAZEK et al., "Identification of Mixed HIV-1/HIV-2 Infections in Brazil by Polymerase Chain Reaction", AIDS, Nov. 1991, 5(11):1293-1299	
		VAN GEMEN et al., "The One-Tube Quantitative HIV-1 RNA NASBA: Precision, Accuracy, and Application", PCR Methods Appl., Feb. 1995, 4(4):S177-S184	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Unique citation designation number. [2] Applicant is to place a check mark here if English language Translation is attached.

PD: 11.03. 1993
P: 1171- 1178 (8)

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Secondary structure of the HIV-2 leader RNA comprising the tRNA-primer binding site

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ABSTRACT

The initiation of reverse transcription of a retroviral RNA genome occurs by a tRNA primer bound near the 5′ end of the genomic RNA at a position called the primer-binding site (PBS). To understand the molecular basis for this RNA-RNA interaction, the secondary structure of the leader RNA of the human immunodeficiency virus type 2 (HIV-2) RNA was analyzed. *In vitro* synthesized HIV-2 RNA was probed with various structure-specific enzymes and chemicals. A computer program was then used to predict the secondary structure consistent with these data. In addition, the nucleotide sequences of different HIV-2 isolates were used to screen for the occurrence of co-variation among putative base pairs. The primary sequences have diverged rapidly in some HIV-2 isolates, however, some strikingly conserved secondary structure elements were identified. Most nucleotides in the leader region are involved in base pairing. An exception is the PBS sequence, of which 15 out of 18 nucleotides are exposed in an internal loop. These findings suggest that the overall structure of the HIV-2 genome has evolved to facilitate an optimal interaction with its tRNA primer.

INTRODUCTION

The 5′ leader region of retroviral RNA contains multiple sequences important for viral replication. These sequences do not code for proteins but are the *cis*-acting sites of recognition by proteins and RNAs responsible for mediating several phases of the viral life cycle. Reverse transcription of the retroviral genome, for example, is primed by a tRNA bound to an 18-nucleotide long, complementary region near the 5′ end of the genome. This region is termed the primer-binding site (PBS) (reviewed in 1–3). Furthermore, processes such as mRNA splicing, polyadenylation and translation are controlled by sequence elements in the leader transcript (reviewed in 2). Another leader region, termed Ψ, is necessary for the selective encapsidation of viral genomes into assembling virions (2). Although specific nucleotide sequences are involved in some of these processes, it may also be that the secondary or tertiary structure of the RNA leader is important. For instance, the human and simian immunodeficiency viruses (HIV and SIV) contain an additional *cis*-acting RNA element at the 5′ end of their RNA, the so-called TAR element (reviewed in 4). TAR forms the target for the transcriptional *trans*-activator protein Tat. Extensive mutational analyses indicated that both specific sequence and structure elements are critical for TAR RNA function (5–8).

Reverse transcription is mediated by the virion-associated enzyme reverse transcriptase (RT). Like all DNA polymerases, RT needs a primer with a free 3′ OH (hydroxyl). All retroviruses utilize a cellular tRNA molecule as primer, its identity depending on the particular virus species. In each instance 16–19 nucleotides at the 3′-CCA end of the tRNA are the exact complement of the corresponding viral PBS. Most mammalian retroviruses use proline tRNA, and avian retroviruses make use of tryptophan tRNA (1,2,9). Sequence analysis of the HIV-1 and HIV-2 viruses revealed a PBS corresponding to lysine tRNA (10), as is the case in other lentiviruses and the mouse mammary tumor virus. The temporal relationship of the association of the tRNA primer with the PBS relative to virus assembly, budding, genome dimerization, Gag protein maturation, *de novo* infection, and reverse transcription has not been rigorously characterized (2). Incorporation of the proper tRNA molecule into the virion was suggested to be the result of a specific interaction with the RT enzyme (11–14). However, these results were obtained with avian retroviruses and could not be reproduced with murine retroviruses (11,15). For the HIV-1 RT protein conflicting data on the specificity of $tRNA^{Lys,3}$ binding were published (16–18). To complicate matters even further, binding of the tRNA to the viral genome was suggested to be catalyzed by the nucleocapsid Gag protein (19). It is nevertheless reasonable to assume that, besides a potential role for the RT and Gag proteins, a critical role in tRNA selection is played by the PBS sequence itself.

There is some evidence for avian Rous sarcoma virus that the structure around the PBS plays a role in initiation of reverse transcription. A potential RNA structure for this region was proposed by Cobrinik *et al* (20,21, see also 22,23). Mutations outside the actual PBS sequence which disrupt this structure impair reverse transcription in infected cells, while mutations which alter the sequence but retain the stem structure have no effect. Studies with permeabilized viruses show that disruption

of the RNA structure does not affect the amount of tRNA primer bound to the viral RNA (20). However, a decrease in the incorporation of the first deoxynucleotides from the 3′ OH terminus of the tRNA primer was measured. In an effort to understand the structural requirements of the PBS region in reverse transcription, we have biochemically analyzed the secondary structure of the RNA leader of human immunodeficiency virus type 2 (HIV-2). We previously reported on the structure of the TAR element at the extreme 5′ end of the HIV-2 genome (24), here we probed a region extending 400 nucleotides into the leader transcript.

MATERIALS AND METHODS

Preparation of RNA

Two plasmids were constructed to facilitate transcription of HIV-2 RNA by the T7 RNA polymerase. Plasmid Blue-TAR2 contains a PstI-KpnI fragment of the HIV-2 molecular clone pROD10 (gift of Dr K.Peden) ligated downstream of the T7 promoter in the polylinker of Bluescript KS(+). This construct was previously described in detail (24) and encodes the HIV-2 leader up to position +420. Plasmid Blue-Gag2 contains the complete HIV-2 leader region (up to the PstI restriction site at position +886) in the correct orientation downstream of the T7 promoter in Bluescript KS(+). Both plasmids were linearized by restriction enzyme digestion in the plasmid sequences downstream of the HIV-2 insert (PvuII-digested Blue-TAR2 and EcoRI-digested Blue-Gag2). T7 transcripts were synthesized according to standard methods (25). Upon DNase treatment and deproteinization of the samples by phenol extraction, the RNA was recovered by ethanol precipitation, dissolved in renaturation buffer (10mM Tris−HCl pH7.5, 100mM NaCl) at approximately 1μg/μl and incubated at 72°C for 2 minutes, followed by slow cooling to 20°C.

Chemical modification and RNase treatment

The renatured RNA (1 μg per reaction) was treated with increasing amounts of the ribonuclease T1 (0−0.001−0.005−0.025 Units), cobra venom nuclease CV (0−0.001−0.003−0.010 Units) or nuclease S1 (0−0.1−0.3−0.9 Units) as previously described (24). After an incubation for 5 min at 37°C, the RNA sample was diluted in 100 μl TE (10 mM Tris−HCl pH7.5, 1mM EDTA), phenol-extracted and recovered by ethanol precipitation. HIV-2 RNA (1 μg in 200 μl) was treated with the single-strand specific chemicals diethylpyrocarbonate (DEP; 0−1-2−4 μl), dimethylsulfate (DMS; 0−0.6−1.2−1.8 μl) or kethoxal (Ket; 0−4 μl). Upon the addition of 10 μg carrier tRNA, the modified transcript was recovered by ethanol precipitation.

Primer extension analysis

The following DNA oligonucleotides were used to map the modified RNA positions in a primer extension reaction (the complementary HIV-2 coordinates are indicated): HIV-2 U5 5′ AGGAGAGATGGGAGCAC 3′ (+183 to +199) HIV-2 PBS 5′ CTGTTCAGGCGCCAACCT 3′ (+299 to +316) HIV-2 psi 5′ TCCGTCGTGGTTTGTTCCTGC 3′ (+373 to +393) Gag-2 AUG 5′ TCTCAAGACGGAGTTTCTCGCGCCCAT 3′ (+546 to +572) Blue-T3 5′ ATTAACCCTCACTAAAG 3′ (complementary to Bluescript sequences in Blue-TAR2 transcripts). Primers were end-labeled with [γ-^{32}P]ATP and T4 kinase. The labeled oligonucleotide (approximately 0.2 ng) was mixed with 0.2 μg modified transcript in a total volume of 12 μl annealing buffer (83 mM Tris−HCl pH 7.5, 125 mM KCl) and incubated for 2 min at 85°C, 10 min at 65°C and slowly cooled down to 25°C. 1 μl (200 Units) of Mo-MuLV reverse transcriptase and 6.5 μl RT buffer (9 mM $MgCl_2$, 30 mM DTT, 150 μg/ml Actinomycin D and 1.5 mM of each dNTP) were added, followed by a 5 min incubation at 37°C and 5 min at 42°C. Upon ethanol precipitation, the samples were taken up in 5 μl formamide sample buffer, denatured at 90°C and analyzed in a 6% acrylamide- 8 M urea gel. The end-labeled primers were also used in dideoxy-sequencing of the appropriate plasmid.

RESULTS

Chemical and enzymatic probing of HIV-2 RNA structure

In vitro synthesized HIV-2 leader RNA was treated with structure-specific probes, followed by primer extension analysis to localize the sites of modification or cleavage. Nucleotides sensitive to RNase S1 or RNase T1, as well as nucleotides that were modified with kethoxal (Ket), dimethyl sulfate (DMS) or diethyl pyrocarbonate (DEP), are assumed to not be involved in base-pairing or base-stacking interactions. Bases that reacted with cobra venom nuclease (CV) were taken to be either in double-stranded regions or single-stranded, stacked regions. The sites of modification were identified by primer extension analysis using

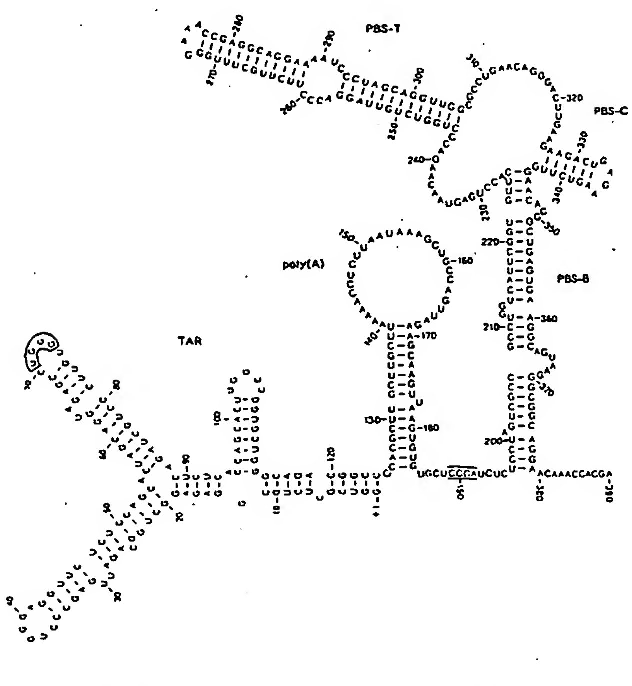


Figure 1. Model of the secondary structure of the HIV-2 leader RNA. Shown is the TAR element at the extreme 5′-end of the transcript (position +1 to +124), the hairpin containing the polyA signal AAUAAA (position +151 to +156) and the region around the PBS sequence (position +303 to +320), which is the perfect complement of the 3′-end of tRNA^{Lys,3}. A putative long-distance interaction between nucleotides in the second TAR loop (5′UGGG3′, position +71/+74) and nucleotides in between the polyA and PBS structures (5′CCCA3′, position +189/+192) is indicated by boxes. Consistent with this proposal, both regions were surprisingly insensitive towards the various single-strand reagents (Figure 2 and 7, reference 24). We like to note that while all leader sequences are present in the 5′ end of HIV-2 RNA, only sequences up to position +172 are present in the repeat element at the 3′ end.

several DNA primers positioned along the HIV-2 leader. The digestion data are summarized in Figures 4–7A, representative experiments are shown in Figures 2 and 3.

The result of all structure mapping experiments is the RNA structure model presented in Figure 1. This region encompasses the TAR element (position +1 to +124), of which the secondary structure was previously determined (24), the polyadenylation signal AAUAAA (position +151 to +156), and the 18 nucleotides of the PBS (position +303 to +320). For all regions of the leader, information on the secondary structure was obtained using at least two different primers and found to be comparable. In addition, we analyzed two HIV-2 transcripts that differ in their 3′ sequences: TAR-2 transcripts contain HIV-2 leader sequences from position +1 to +420, while Gag-2 transcripts run into the *gag* open reading frame (+1 to +886, including the *gag* startcodon at position +546). The results of the structure probing were comparable for these two RNA species. In the next sections, we will deal with the different structural domains depicted in Figure 1 in detail.

The polyA hairpin (position +125 to +184)

Treatment with several single-strand reagents (DEP,DMS,S1,T1) produced strong bands in the +140/+170 region, but not in the immediate upstream and downstream sequences (Figure 2). The results are summarized in Figure 4A, in which the locations of the nucleotides that were modified under native conditions are superimposed on the RNA secondary structure model. A stem-loop hairpin structure consistent with the digestion pattern can be drawn that presents the polyA signal (position +151/+156) in the single-stranded loop. We will refer to this structure as the polyA hairpin. Cobra venom nuclease reactivity occurs at multiple positions on both sides of the proposed single-stranded region, suggesting that these regions may be stacked on top of the base-paired stem. Two A-rich stretches are present in the loop; AAAAA at position +140/+144 and the polyA signal AAUAAA at position +151/+156. Interestingly, both stretches are highly reactive towards the chemicals DMS and DEP, while only the latter is susceptible to RNase S1 treatment. Because extensive base-stacking was observed in the AAAAA region, but not in the AAUAAA region, it is possible that base stacking or steric hindrance blocks access of the S1 enzyme.

Secondary structure predictions based on the primary sequence of the polyA region were made using several computer algorithms (26,27). Both programs predict the polyA hairpin, even without the inclusion of the modification data of Figure 4 as a restrictive parameter. The only difference is additional base-pairing between the 5′ and 3′ side of the loop (5′AAC3′ paired to 5′GUU3′), which is not consistent with the experimental data (Figure 4A).

Sequence comparison of RNA from different HIV-SIV isolates has been very instructive in solving the TAR RNA structure (24,28). We performed a similar phylogenetic analysis for the polyA region using nucleotide sequences from thirteen retroviruses (10). Besides seven HIV-2 isolates (Figure 4B, numbers 1–7), we included sequences from the closely related simian immunodeficiency viruses isolated from macaques and sooty mangabeys (SIV-MAC and SIV-SMM, numbers 8–11 and 12–13, respectively). The RNA structure of the polyA region is generally supported by the phylogenetic analysis (Figure 4B). The majority of the nucleotide substitutions observed were in the single-stranded loop and bulge regions and therefore do not affect

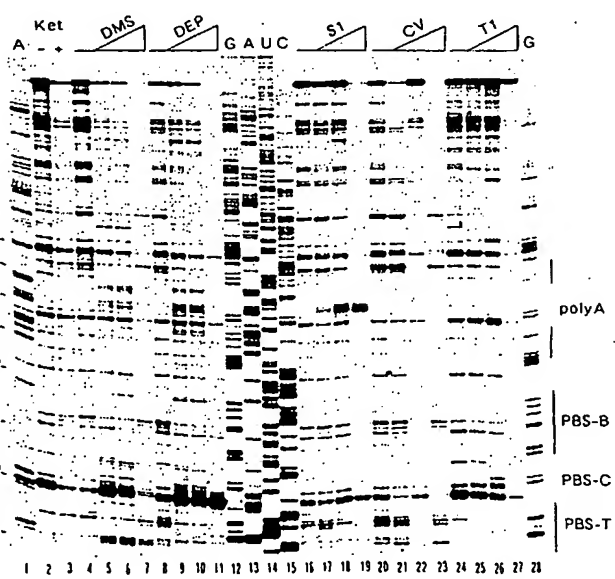


Figure 2. Nuclease digestion and chemical modification of TAR-2 RNA under native conditions. *In vitro* transcribed HIV-2 leader RNA was treated with limiting amounts of several single-strand specific reagents (Ket=kethoxal (G-specific), DMS=dimethyl sulfate (A,C-specific), DEP (diethyl pyrocarbonate (A-specific), S1=RNase S1, T1=RNase T1 (G-specific)) and the double-strand-specific RNase CV (Cobra Venom). Each treatment (indicated above the lanes) was performed with increasing amounts of the chemical-RNase (see materials and methods for details, left lanes represent mock incubations). Cleavage-modification sites were detected using primer-extension analysis with the HIV-2 psi primer. Electrophoresis was on 6% polyacrylamide/7M urea gels. For reference, the psi primer was used in a DNA sequence reaction (lanes 12–15; GAUC and lane 1 (A) and 28 (G). Numbers on the left represent HIV-2 coordinates (+1 being the transcription start site). The position of double-stranded stem regions is schematically indicated

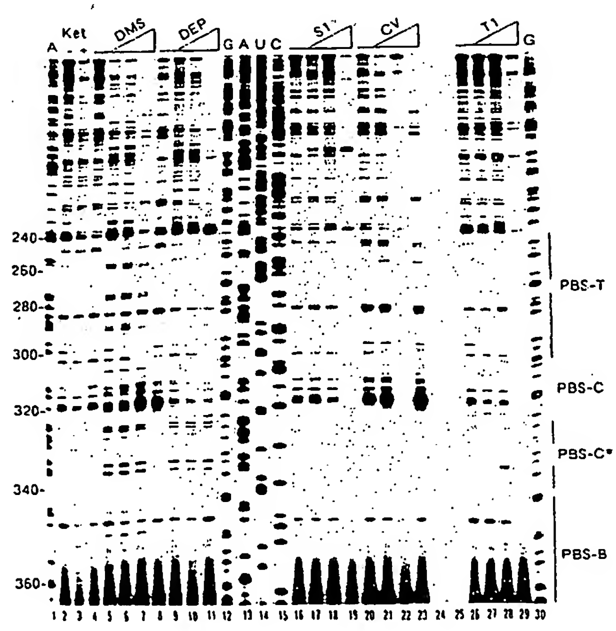


Figure 3. Nuclease digestion and chemical modification of TAR-2 RNA under native conditions. See legend to Figure 2 for details. The position of the different leader regions is schematically indicated on the right. The small hairpin in the central PBS domain is indicated by PBS-C*.

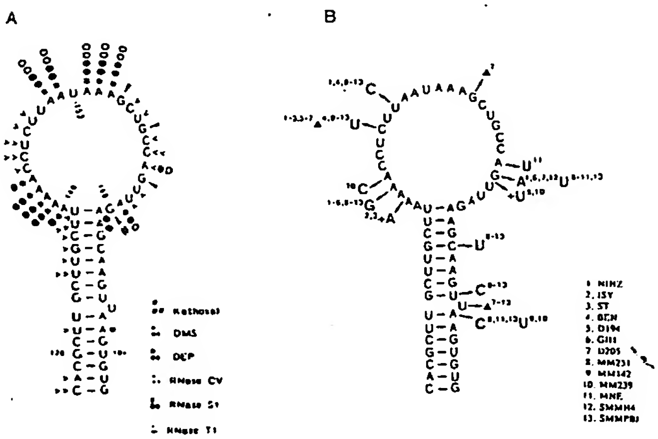


Figure 4. Secondary RNA structure model for the polyA region; summary of the digestion data (A) and phylogenetic analysis (B). Sensitivities of the RNA to the various reagents are indicated by symbols (see insert). The sequence/structure of the HIV-2 ROD isolate is shown as prototype and compared to 13 different isolates (1−7=HIV-2; 8−11=SIV-MAC; 12,13=SIV-SMM). Nucleotide changes occurring in these isolates are indicated in bold with the number of the particular isolate in superscript. Deletions are shown as ▲, insertions are indicated by a + sign.

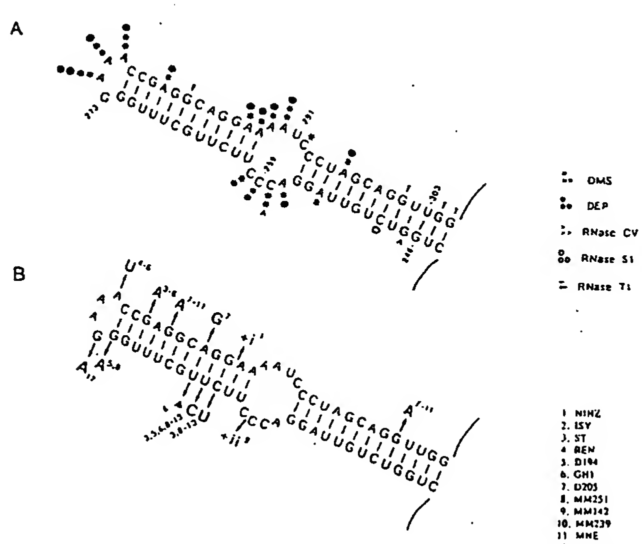


Figure 5. Secondary RNA structure model for the PBS-T region; summary of the digestion data (A) and phylogenetic analysis (B). See legend to Figure 4 for details. Two major insertions are indicated in the internal loop region (i=5'CGG3', ii=5'CUGGUCUGUUAGGACC3').

base-pairing. Furthermore, two 1-nucleotide insertions and two 1-nucleotide deletions were present in the loop. Most sequence changes in the stem area do not disturb the basepairing scheme either. Interestingly, six isolates (8−13) mutate a G-U basepair into the more stable G-C pair, and at the same time change a G-C into the less stable G-U pair. Conservation of stem stability can also explain the alterations seen for many isolates in the bulge loop. A deletion of the 1-nucleotide bulge (isolates 7−13) is

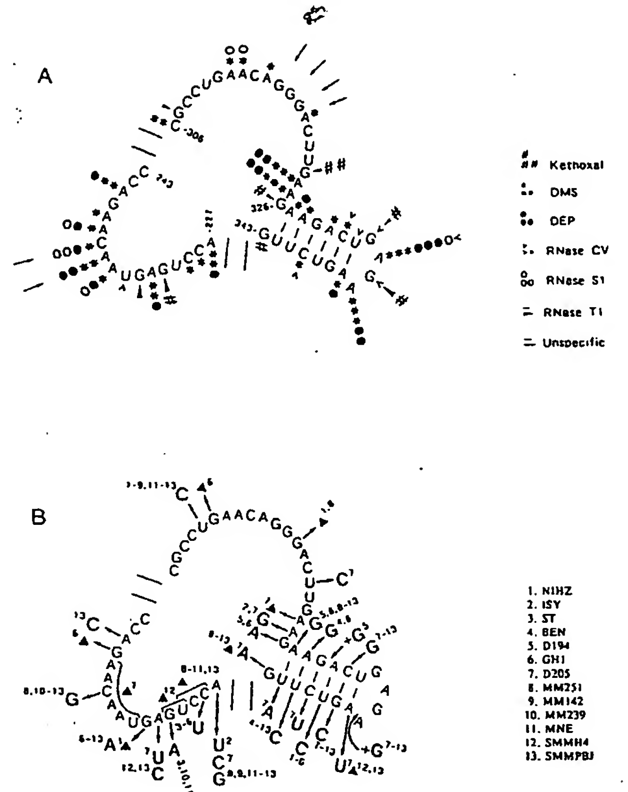


Figure 6. Secondary RNA structure model for the PBS-C region; summary of the digestion data (A) and phylogenetic analysis (B). See legend to Figure 4 for details. Positions at which strong, non-specific reverse transcriptase stops were observed are indicated by arrows.

compensated in most viral genomes by the generation of a mismatch at the U-A basepair flanking the bulge (isolates 8−11,13). These combined results suggest that the overall stability of this 15-basepair hairpin is kept within narrow limits.

The structure of the PBS region (position +197 to +379)

A combination of the three forementioned approaches (structure probing, computer analysis and comparative sequence analysis) was needed to derive the structure for the PBS region shown in Figure 1. We will separately deal with the different domains; the top part consisting of an extended stem-loop structure (PBS-T, Figure 5), the relatively unstructured central domain consisting of a large internal loop and a small stem-loop element (PBS-C, Figure 6), and the bottom part in which leader sequences around position +210 are annealed to sequences around +360 (PBS-B, Figure 8).

The PBS-T region

The structure shown was predicted by the algorithm for RNA folding and is strongly supported by the experimental structure analysis (Figure 2 and 3, results are summarized in Figure 5A). Most informative are the DMS and DEP treatments that show highly susceptible nucleotides in the loop (position +274/+276) and internal loop (+257/+260 and +287/+292) domains. Some RNase T1 reactivity was observed on the 3'-side of the lower stem region (positions +301, +304 and +305). Interestingly, this region contains four consecutive G-U basepairs that might destabilize the helix (see discussion).

For a rigorous phylogenetic analysis, there should be dual base changes such that the alteration of the primary sequence at two residues in a stem will be compensatory to retain structure (e.g. G-C to A-U). Among the thirteen HIV-SIV isolates that were analyzed, no such dual base changes were observed for the PBS-T region (Figure 5B). However, many of the substitutions do conserve basepairing (e.g. U-G to U-A at position +279 and +281). For seven isolates (3, 8−13), a stem-destabilizing change at position +263 (C-G to U-G) was compensated for by a structure-stabilizing mutation in the next basepair (U-G to C-G, position +264). Major differences do occur in the sequences of isolate 9 (a 16-nucleotide sequence duplication at position +259) and isolate 1 (a 3-nucleotide insertion at position +286). Because of their location in or close to the internal loop area, these changes are not expected to disrupt the overall stem-structure.

The PBS-C region

The central domain of the PBS structure (PBS-C, Figure 6) consists of a 6-basepair stem-loop element (position +327/+343) and a relatively large internal loop (17 nucleotides at the 5′ side [position +227/+243] and 21 nucleotides at the 3′ side [position +306/+326]). The 5′ side of the internal loop is characterized by high susceptibility to all single-strand specific reagents (Figure 6A) and a dramatic variability in sequence among the different HIV-SIV isolates (Figure 6B). In fact, all nucleotides except two are mutated or deleted in one or more viral isolates. This invariant dinucleotide is the CC sequence adjacent to the PBS-T stem (position +242/+243). Given these results, combined with the inability to pair the +227/+243 region to any other sequence in the HIV-2 leader (either by computer analysis or by hand), we believe this region to not be involved in hydrogen bonding.

The analysis of the 3′ side of the internal loop was different from that of the 5′ side in that less reactivity was observed (Figure 6A), and that only a few base substitutions-deletions were detected (Figure 6B). The absence of major nucleotide changes is easily explained by the important function of this region as a primary sequence element; at least 15 nucleotides (position +306/+320) constitute the PBS, which is complementary to the $tRNA^{Lys,3}$ primer. Analysis of the complete HIV-2 leader sequences did not reveal any possibility for basepairing of this region. In addition, the reactivity pattern of this region was independent from the actual 3′ end of the HIV-2 sequences (either +420 or +886). The combined results suggest that this region is not highly structured. In the absence of phylogenetic data, we have therefore drawn this region as single-stranded. At this moment, however, we cannot vigorously exclude local basepairing (e.g. CCU310-GGG318), interactions within the internal loop (e.g. CCCUG246-GGGAC314), or tertiary interactions that limit the accessibility of this region.

The structure of the small hairpin in the central domain was suggested by the digestion experiments (Figure 3, PBS-C* area, results are summarized in Figure 6A) that showed major reactivity in the tetranucleotide loop area (position +333/+337). However, the presence of this hairpin was initially corroborated neither by the computer analysis, nor by the comparative sequence analysis (Figure 6B). At first glance, the existence of the helical stem is supported by only one compensatory base change (C-G to G-C for isolates 7−13), while multiple mutations are expected to disrupt the stem at most other basepair positions. A closer look at the individual sequences, however, does reveal that all isolates can adopt a similar stem-loop structure through

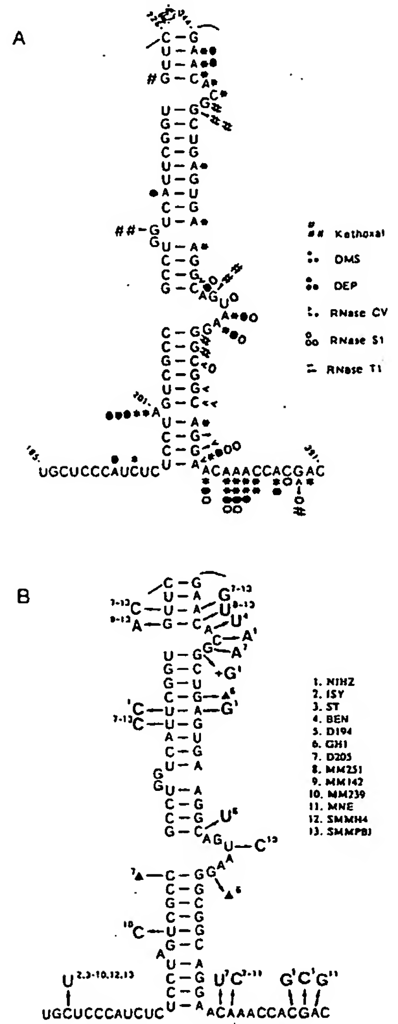


Figure 7. Alternative stem-loop structures in the region 3′ of the PBS. The dramatic nucleotide variability seen in this region for most HIV-2 isolates do affect the folding pattern as proposed for the prototype HIV-2 ROD (Figure 6). Alternative stem-loop elements can be formed through realignment of the mutated sequences. Bases that differ from the prototype ROD sequence are circled. The position of deletions (▲) and insertions (+) are indicated. Two alternative folding patterns are shown for both the D194 and the H4/PBJ isolates. Putative hydrogen bonding between A and C is indicated by ●. The possibility of non-Watson−Crick C-A basepairing has been described for tRNA (29), rRNA (30) and phage RNA (31).

realignment of the primary sequences (Figure 8). These results suggest that RNA structure is of primary importance for this region just downstream of the PBS sequence. Interestingly, for all alternative hairpin structures, the conserved pentanucleotide sequence UGAGA is present at the apex of the stem.

The PBS-B region

The structure of the PBS-B region was predicted by computer analysis and confirmed by the digestion experiments (Figure 3, summarized in Figure 7A) and the phylogenetic analysis (Figure 7B). All single-stranded areas were positively identified. For instance, the bulged-out A at position +201 was highly accessible to the chemicals DEP and DMS, but no reactivity of the adjacent nucleotides was scored (Figure 7A). Relatively little

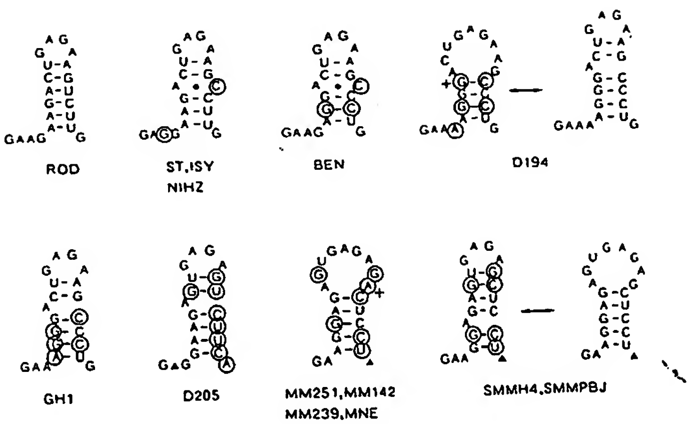
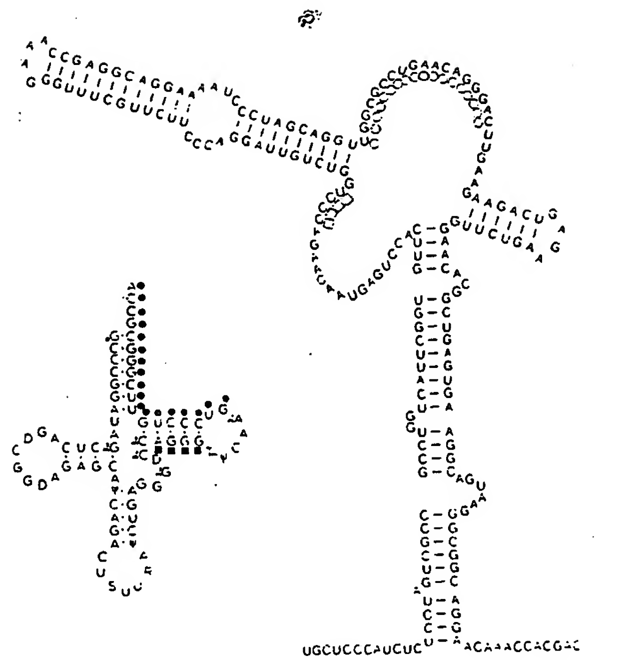


Figure 8. Secondary RNA structure model for the PBS-B region; summary of the digestion data (A) and phylogenetic analysis (B). See legend to Figure 4 for details.

reactivity was observed for the region that connects the polyA and PBS structures (position +185/+196). A potential tertiary interaction of these sequences with upstream sequences in the TAR element may be responsible for this shielding effect (see Figure 1).

Three base changes on one side of the PBS-B stem are compensated by base substitutions in the opposite strand (Figure 7B: the 3rd, 4th and 9th basepair from the top). Thus, the basepairing is conserved even though the sequence is not. Furthermore, the overall stability of the upper part of the stem seemed to be conserved among isolates; the U-A to C-G change for isolates 7–13 was accompanied by a G-C to A-U change at the next basepair.



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Standard tRNA-PBS interaction (●○)

Loss of bp in tRNA:                -12
Loss of bp in PBS-RNA:             - 3
Gain of bp in tRNA-PBS complex:    +18
                                   ----
                                   + 3

Extended tRNA-PBS interaction (●○ +■□)

Loss of bp in tRNA:                -12
Loss of bp in PBS-RNA:             - 3
Gain of bp in tRNA-PBS complex:    +22
                                   ----
                                   + 7
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Figure 9. Secondary RNA structures of the tRNA^{Lys} primer and the HIV-2 PBS region. Compared to Figure 1, part of the PBS-T stem is melted to expose the complete PBS sequence in order to allow an 18-basepair interaction with the 3' end of the tRNA. The initial contact sites are marked by circles (tRNA; filled-in circles, HIV-2 PBS; open circles). The number of basepairs lost and formed during this 'standard' interaction is indicated. Subsequent stabilization of this complex is possible through an additional 8 basepairs between the regions marked by squares (tRNA; filled-in squares, HIV-2 PBS; open squares). The total number of basepairs lost and gained in this 'extended' annealing reaction is shown.

DISCUSSION

Thusfar, limited experimental data are available on RNA secondary structure of retroviral leader sequences (5,20,22–24,32,33). Here, we present a structural model for HIV-2 RNA leader based on the susceptibility of various regions of the molecule to cleavage by nucleases and on phylogenetic comparison of sequences from other HIV-2 viruses (Figure 1). The RNA chain folds into a short polyA stem-loop structure and a much larger, irregular PBS structure. The structure of the first 400 nucleotides of the HIV-2 leader consists of many short helix segments, which are interrupted by a variety of structural irregularities (bulges, internal loops etc.). In general, the stability of the individual helices is maintained relatively constant even though multiple base substitutions do occur among viral isolates. The tendency not to produce perfect stems may reflect the biological role of the RNA molecule as template in reverse transcription and translation. Progression of the RT enzyme and ribosomes, respectively, is best served by the presence of weak helices.

The existence of most stem regions is supported by compensatory base substitutions. The phylogenetic data can be summarized as follows. For basepaired nucleotides, a G to A change is expected to occur more frequently in the context of G-U pairs (G-U to A-U, conservation of basepairing) than G-C pairs (G-C to A C, loss of pairing). Indeed, out of 5 G to A ... he HIV-2 helixes, 3 were in the context of G-U pairs and only 1 G-C pair was altered. The 5th mutation was a co variation (G-C to A-U) that conserved basepairing. This analysi becomes even more striking when one takes into account the tota number of G-U and G-C pairs (14 and 33, respectively). A simila analysis can be performed for U to C changes, that are expecte to predominate in U-G over U-A pairs. Out of a total of 14 U-C pairs, 4 were found to change into C-G. In contrast, no individua U to C change was observed for the 26 U-C basepairs Additionally, 4 co-variations (U-C to C-G) were scored.

One striking feature of the structure model is the predominar single-strand character of the PBS sequences. Only 3 basepair of the PBS-T stem need to open up in order to expose th complete 18-nucleotide PBS (Figure 9, the open circles) Remarkably, this region of the PBS-T stem contains fo

consecutive G-U basepairs and some susceptibility to single-strand reagents was detected (Figure 5A). Thus, melting of this part of the PBS-T stem, in order to facilitate binding of the tRNA 3'-terminal CCA, is not expected to be a major hurdle. In fact, since all tRNA species share the same 3'-terminal CCA sequence, the initial inaccessibility of the GGU complement might contribute to the process of tRNA selection.

Interaction of the primer $tRNA^{Lys,3}$ with the HIV-2 PBS sequence of the retroviral genome involves 18 basepairs. For this intermolecular basepairing to occur, however, the tRNA sequences should not be involved in stable intramolecular basepairing. This implies that part of the cloverleaf structure of tRNA has to be unfolded (Figure 9). A total of 12 basepairs in the tRNA molecule must be sacrificed to allow a complete 18-basepair interaction with the HIV-2 target sequences (Figure 9, the circles in tRNA and PBS molecules). In addition, some tertiary interactions within the DHU loop (T_m and $_mA$) and between nucleotides in the DHU and TΨC loops (ΨC and GG) are likely to be lost at this point. It is possible that binding of the RT enzyme, which was reported to recognize the anticodon domain of $tRNA^{Lys,3}$ (16), does induce a conformational change in the tRNA molecule that enables the acceptor and TΨC stems to open up. The RT protein may also actively promote helix unwinding, as was described for the avian retroviral enzyme on RNA−DNA and DNA−DNA duplexes, but not on RNA−RNA duplexes (34). Alternatively, a role for the Gag nucleocapsid protein in the unwinding of the tRNA structure has been reported (19).

Based on sequence analysis, it has been suggested that other regions of the tRNA molecule can form additional contacts with the retroviral genome (reviewed in 2). Haseltine *et al.* (35,36) reported that 7 bases of the TΨC loop of the $tRNA^{Trp}$ primer could anneal to upstream sequences of the Rous sarcoma virus RNA. Consistent with this idea, Cordell *et al.* (37) showed that a 3'-terminal, 18-nucleotide tRNA fragment lacking the TΨC loop could not efficiently prime the reverse transcription reaction. Extension of this fragment with an additional 9 nucleotides from the TΨC loop did produce an active primer species. Recently, elegant work by Aiyar *et al.* (38) provided experimental evidence for the importance of this putative second interaction site. It was demonstrated that the additional interaction is necessary for efficient initiation of reverse transcription *in vitro* as well as in permeabilized virions. Furthermore, their experiments showed that besides primary sequences also certain structure elements are important for PBS function (20,21,38).

In our structure model of HIV-2 RNA, 8 additional basepairs can be formed between the TΨC loop of $tRNA^{Lys,3}$ (Figure 9, filled-in squares) and nucleotides on the 5' side of the internal PBS loop (open squares). Sequence analysis of this second binding site in a number of HIV-2 and SIV isolates (Figure 5B,6B) indicated that this sequence motif is highly conserved, which is consistent with its proposed role in tRNA binding. Interestingly, the extended tRNA−PBS interaction is possible without the opening up of additional basepairs in the tRNA molecule and only one G-U pair of the PBS-T stem needs to be melted. It is the initial interaction between tRNA and PBS through the 'standard' basepairs (circles in Figure 9) that forces most nucleotides needed for the 'extended' interaction (squares in Figure 9) into the single-stranded form. These sequences (in the TΨC stem of the tRNA molecule and the 5'-side of the HIV-2 PBS-T stem) can subsequently anneal to further stabilize the complex. In this view, interaction between the retroviral genome and its tRNA primer occurs in two steps and the structure of the PBS region is suggested to play an important role in the process of reverse transcription. The secondary structure of HIV-2 RNA brings two regions of the genome into juxtaposition (the 'standard' PBS [circles, position 310] and the 'extended' PBS [squares, position 240]) that together facilitate stable binding of the tRNA primer. Since similar structures and interactions can be found for several retroviral RNAs (38), it is likely that these viruses utilize a common mechanism to initiate reverse transcription.

It should be stressed that we do not believe genomic RNA to be the only viral component needed for tRNA selection and binding. For avian viruses, a specific interaction between the RT enzyme and the tRNA primer was reported (11,12). This idea is supported by the apparent absence of primer from Rous sarcoma virus particles that lack polymerase (13,39) and the inclusion of primer in murine leukemia virus particles that lack viral RNA (40). However, the situation appears to be different in the murine retroviruses where no specific RT−tRNA binding was found (15). This was most conclusively shown by the genetic data of Colicelli and Goff (9). They describe a revertant of the Moloney murine leukemia virus with a PBS no longer specific for the usual $tRNA^{Pro}$, but for $tRNA^{Glu}$. This result suggests that the murine RT protein can utilize different tRNAs and that tRNA selection may be governed by the PBS sequences. Interestingly, the reversion-mutation was not restricted to the 18-nucleotide PBS region. Instead, a much larger genome segment around the PBS was acquired. Given the importance of the secondary, upstream tRNA binding site and the observation that both PBS sites are part of a much larger, specific RNA structure, it seems likely that the $tRNA^{Glu}$-PBS can function only in the appropriate sequence context. For instance, in order for the HIV-2 PBS sequence to fold into the correct secondary structure, a large segment of the sequences flanking the PBS need to be present (Figure 1, position +200 to +380). Further studies on the minimal requirements of the PBS region for primer binding (20,41,42) may reveal more details of this RNA−RNA interaction.

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XX
SV   X72325.1
XX
DT   24-JUN-1993 (Rel. 36, Created)
DT   24-JUN-1993 (Rel. 36, Last updated, Version 2)
XX
DE   Human immunodeficiency virus-2 leader RNA
XX
KW   .
XX
OS   Human immunodeficiency virus 2
OC   Viruses; Retroid viruses; Retroviridae; Lentivirus;
OC   Primate lentivirus group.
XX
RN   [1]
RP   1-390
RX   MEDLINE; 93219077.
RA   Berkhout B., Schoneveld I.;
RT   "Secondary structure of the HIV-2 leader RNA comprising the tRNA-primer
RT   binding site";
RL   Nucleic Acids Res. 21:1171-1178(1993).
XX
FH   Key             Location/Qualifiers
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FT                   /db_xref="taxon:11709"
FT                   /organism="Human immunodeficiency virus 2"
XX
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     ggcagtaagg gcggcaggaa caaaccacga                                        390
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Detection of Human Immunodeficiency Virus Type 1 RNA in Plasma Samples from High-Risk Pediatric Patients by Using the Self-Sustained Sequence Replication Reaction

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There is an urgent need for rapid and sensitive methods to assess human immunodeficiency virus (HIV) infection in infants and children. We evaluated an approach by using the self-sustained sequence replication reaction (3SR) to amplify HIV type 1 (HIV-1) RNA directly. The amplified RNA product was then detected by bead-based sandwich oligonucleotide capture hybridization and rare earth metal chelate time-resolved fluorescence. The sensitivity of this technology was determined to be less than 12 HIV-1 RNA copies with an amplification level of 10^{10}-fold with purified HIV-1 RNA. Plasma samples from 19 high-risk pediatric patients younger than 5 years of age were examined, and results were compared with viral culture of patient plasma. Results from plasma culture and 3SR amplification agreed for 14 of these patients and disagreed for 5. Of the five samples which did not agree, four were positive by 3SR and negative by culture and one was positive by culture and negative by 3SR but became positive by 3SR at a subsequent testing. We conclude that 3SR amplification coupled with time-resolved fluorescence is a promising technology for investigating the relationship between the presence of HIV-1 RNA in plasma and progression of disease in HIV-infected pediatric patients. This technology should be important in the assessment of HIV-1 infection, in evaluating drug therapies, and in understanding the pathogenesis and transmission of the virus.

A major problem in evaluating a human immunodeficiency virus type 1 (HIV-1) infection in pediatric patients is that there are no standard methods to assess the extent of disease and efficacy of therapy. Maternal transfer of antibody across the placenta during gestation results in virtually all infants born to HIV-infected mothers being seropositive at birth; however, only 30 to 50% of these babies are actually infected with the virus (2), and determining changes in antibody titer has very limited value. The p24 antigen assay as well as other markers, such as CD4 counts, beta 2 microglobulin, neopterin, and the symptomatology used to assess HIV-1-infected adults, are generally unreliable in infants (2).

In adults, HIV-1 can be isolated from both peripheral blood mononuclear cells (PBMCs) and plasma. The success rate of isolation is usually much higher for PBMCs than plasma (1). Ehrnst et al. (10) reported the isolation of HIV-1 during all stages of infection in adults, with highest isolation (100%) during the AIDS stage and lowest (~50%) during the asymptomatic stage. Ho et al. (13) also found a strong correlation between the stage of disease and the titer of HIV in the plasma but not the titer of virus in PBMCs.

Because of the increasing number of infected infants born to HIV-1-infected mothers, the early onset of clinical symptoms, the high mortality rates in infants, and because there are no good markers for the extent of HIV disease in pediatric patients, the development of nucleic acid amplification systems such as the polymerase chain reaction (PCR) (11, 17) and RNA transcription-based systems, including the transcription-based amplification system (TAS) (15) and the self-sustained sequence replication (3SR) reaction (12), lend promise to the sensitive detection and assessment of HIV-1 infection in these patients. The TAS and PCR amplification systems were recently compared for sensitivity of detection of HIV-1 sequences in 86 PBMC specimens from 30 HIV-1-positive patients. TAS and PCR had similar detection sensitivities: 93 and 95%, respectively (8). In this paper we report the development and evaluation of a system that uses 3SR amplification, bead-based sandwich capture hybridization (23), rare earth metal chelate-labeled probes (18), and time-resolved fluorescence (6, 7) for direct detection of HIV-1 RNA in plasma. These results were compared with viral culture of patient plasma.

MATERIALS AND METHODS

Specimens. Thirty 3- to 5-ml samples of heparin-anticoagulated peripheral blood were drawn from 19 HIV-1 high-risk pediatric patients (five were newborns, eight were <1 year, three were <2 years, and three were 3 to 5 years old) at the University of California Davis Medical Center. All patients were born to seropositive mothers. Plasma was separated from cells by centrifugation at 800 × *g* for 15 min. Plasma was further clarified by centrifugation at 950 × *g* for 30 min and then placed in plasma culture or tested for the presence of viral RNA by using 3SR amplification.

Plasma culture. One milliliter of patient plasma was mixed with 10^7 phytohemagglutinin (Difco, Detroit, Mich.)-stimulated PBMCs from HIV-1-seronegative donors. Lympho-

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TABLE 1. Sequences of HIV-1 synthetic oligonucleotides

Oligonucleotide	Orientation	Sequence (5'-3')	Position[a]
Primer			
1	Sense	GGT TTT GCG ATT CTA	6465–6479
2	Antisense	(AAT TTA ATA CGA CTC ACT ATA GGG A)[b] TAG CAT TGT CTG TGA	6631–6645
Probe	Sense	(Aminolink-TTTTT)[c] AAT TAG GCC AGT AGT ATC AAC TCA ACT GCT	6551–6580
Capture	Sense	(Aminolink-TTTTT)[c] AGT CTG GCA GAA GAA GAG GTA GTA ATT AGA	6591–6620

[a] Ratner et al. (19).
[b] T7 promoter sequence.
[c] Aminolink coupling agent for chelate and bead attachment and 5-T-base spacers.

cytes were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 20 U of interleukin 2 (Cellular Products, Buffalo, N.Y.) per ml, 2 μg of polybrene (Sigma) per ml, 5×10^{-5} M 2-mercaptoethanol (Sigma), and 10 U anti-alpha interferon (ICN Immunobiologics, Lisle, Ill.) per ml. The supernatants were tested biweekly for HIV-1 p24 antigen by using a commercial solid-phase sandwich enzyme-linked immunosorbent assay (E. I. DuPont, Wilmington, Del.). A culture was considered positive when the absorbance values of the test were more than two times the values for samples from seronegative PBMCs and when positive values were noted on at least three consecutive samplings, indicating viral replication. Supernatants from cultures of HIV-1-negative plasma were consistently negative when tested by the antigen assay. Cultures reported negative were held for 4 weeks.

Preparation of RNA for amplification. RNA was isolated from the plasma samples as described by Chomczynski and Sacchi (4), with minor modification. Briefly, 100 μl of clarified plasma was mixed with 1.0 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0)–0.5% sarcosyl–0.1 M 2-mercaptoethanol)–100 μl of 2 M sodium acetate, pH 5.0–1.0 ml of water-saturated phenol–200 μl of chloroform-isoamyl alcohol (49:1). The solution was vortexed briefly and incubated on ice for 15 min. The RNA fraction was recovered by centrifugation at 10,000 × g for 20 min at 4°C and was followed by ethanol precipitation of the aqueous layer. RNA was pelleted by centrifugation at 16,000 × g for 20 min at room temperature. To ensure high purity, the RNA pellet was resuspended in solution D, ethanol precipitated, dried, and resuspended in 0.1% diethylpyrocarbonate-treated water.

Synthetic oligonucleotides. The sequences of the oligonucleotides used are given in Table 1. Sequences were derived from the complete nucleotide sequence of the HIV-1 genome (19). The DNA oligomers were chemically synthesized on a Applied Biosystems DNA synthesizer (model 380 B) and were purified by using a Poly-Pak oligonucleotide purification cartridge (Glen Research, Sterling, Va.). The envelope-specific primers used in this study have been successfully used by others to amplify a 298-base RNA fragment of HIV-1 by 3SR amplification (12). The target-specific probe is complementary to a nonprimer portion of the amplified product.

3SR amplification. Figure 1 summarizes the steps involved in the 3SR reaction. This amplification reaction was performed in a total volume of 50 μl containing 5 to 10% of the total extracted patient plasma RNA or 4.0 μl of diluted and purified HIV RNA (dilution range, 10^4 to 10^{-1} copies), 0.125 μg of each primer, 1 mM (each) the four deoxynucleoside triphosphates, 6 mM (each) the four ribonucleoside triphosphates, 40 mM Tris-HCl (pH 8.1), 30 mM $MgCl_2$, 20 mM KCl, 4 mM spermidine trihydrochloride (Sigma), 10 mM dithiothreitol, 10% dimethyl sulfoxide, and 15% sorbitol. All stock solutions and diluted reagents were made in diethylpyrocarbonate-treated water. Amplification reactions were heated to 65°C for 2 min followed by temperature equilibration to 42°C. AMV reverse transcriptase (15 U) (Life Sciences, St. Petersburg, Fla.), T7 RNA polymerase (50 U) (Stratagene, La Jolla, Calif.), and *Escherichia coli* RNase H (3 U) (Bartels Division, Baxter Diagnostics Inc., Seattle, Wash.) were added. Enzymes were mixed by gentle pipetting, and the reaction tubes were incubated at 42°C for 90 min. After amplification, the tubes were placed on ice for approximately 10 min and briefly spun in a microcentrifuge.

Preparation of labeled probes. The rare earth metal chelate label was functionalized for attachment to the oligonucleotide probe by dissolving 7.0 mg of the reagent in 100 μl of 0.2 M LiOH. To this solution was added 100 μl of 0.5 M sodium carbonate and 200 μl of water. Five microliters of thiophosgene was added in chloroform to the chelate solution. The solution was stirred and then dried under reduced pressure. Twenty optical density units (57 nmol) of the 30-base oligonucleotide probe with a 5-thymidine-base spacer and functionalized on the 5' end with 6-aminohexyl phosphate (aminolink) were dissolved in 0.1 M sodium borate, pH 9.5. The chelate solution and oligomer solution were combined and reacted for 12 h with constant stirring. The probe was purified by using reverse-phase high-pressure liquid chromatography which was followed by gel filtration. Purified probe was verified by 14% polyacrylamide gel electrophoresis followed by terbium staining of the chelate and ethidium bromide staining of the DNA. These staining reactions ensured that the probe preparation was uncontaminated with unlabeled DNA probe. The specific activity of the probe (2000 time-resolved counts per fmol) was determined by directly capturing 1 and 10 fmol of probe on complementary beads. The average time-resolved counts per femtomole was calculated by subtracting the background and dividing by the number of femtomoles used in duplicate direct capture assays.

A radioactive oligonucleotide probe was made by 5' end labeling with [γ-^{32}P]ATP (Amersham, Arlington Heights, Ill.) by using T4 polynucleotide kinase (Stratagene) as described by Sambrook et al. (20) Unincorporated label was removed from the labeled probe by using G-50 nick columns (Pharmacia, Uppsala, Sweden).

Capture bead preparation. Preparation of polystyrene capture beads (Seradyn, Indianapolis, Ind.) followed the protocol described by Lund et al. (16), with slight modification. Briefly, 200 μl of carboxymethyl latex beads (0.8 μm) were prewashed three times in distilled water. After the final

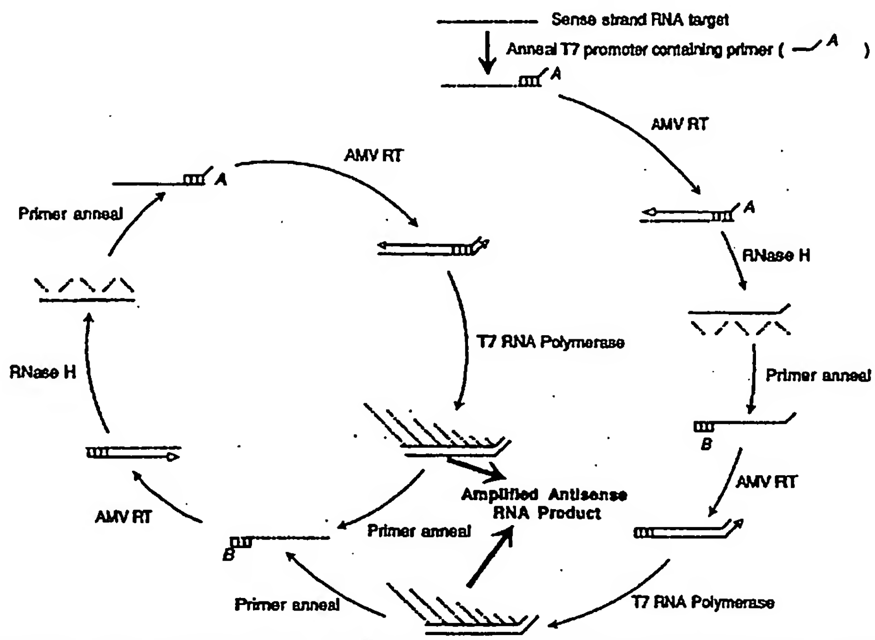

FIG. 1. 3SR amplification reaction. 3SR utilizes simultaneous reverse transcription and RNA transcription to produce multiple RNA copies of the target sequence by means of cDNA intermediates. Oligonucleotide primers A and B are used to prime cDNA synthesis, with primer A containing the promoter sequence required by the T7 RNA polymerase molecule. cDNA synthesis requires the digestion of RNA in the RNA-DNA duplex by the enzyme RNase H. The cDNAs are then used to produce RNA copies of the target sequence. The target RNAs can then further serve as templates for additional amplification of the target sequence.

wash, the beads were resuspended in 0.2 ml of 0.1 M imidazole buffer, pH 6.0. The bead suspension was mixed with 20 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 0.6 optical density units of capture oligonucleotide resuspended in 0.1 M imidazole buffer, pH 6.0. The beads were allowed to react overnight with gentle agitation and then washed four times in 1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate. Beads were resuspended in wash buffer and stored at 4°C.

Bead-based sandwich assay. Aliquots of the 3SR amplification mixture (1.0 to 10.0 μl) were analyzed by bead-based sandwich capture hybridization. The hybridization solution (50 μl) contained 10 μl of a suspension of capture polystyrene beads (5 μg/μl) to which was bound the HIV-1 complementary 30-base capture sequence, 0.75 M NaCl, 0.075 M sodium citrate, 25 mM morpholinepropanesulfonic acid (MOPS) pH 7.0, and either 100 fmol of rare earth metal chelate-conjugated oligonucleotide probe or 100 fmol of ^{32}P-labeled probe. The hybridization solution was pipetted into a microtiter plate (Pandex Division, Baxter Diagnostics Inc., Mundelein, Ill.) (or microfuge tube for the ^{32}P-labeled probe control assay), covered, and incubated at 50°C for 60 min. The beads were washed twice at room temperature with 0.1× SSC. A solution of 3.5 μM terbium in 0.2 M magnesium chloride and 1.5 μM EDTA was added to the beads and incubated for 10 min at room temperature. Beads were read in either a fluorescence particle concentration (14) time-resolved instrument (Baxter MicroScan Research Instrument) at the excitation wavelength of 340 nm and the emission wavelength of 545 nm or a liquid scintillation counter (Beckman, Fullerton, Calif.). Figure 2 illustrates the principles of the bead-based sandwich time-resolved fluorescence assay for the detection of HIV-1.

RESULTS

Sensitivity. The sensitivity of the 3SR procedure was determined by amplifying decreasing amounts of purified HIV-1 RNA (Fig. 3). By using 3SR amplification, the bead-based sandwich capture assay and a rare earth metal chelate-labeled probe or a ^{32}P-labeled probe, less than 12 copies of a 298-base RNA fragment could be detected. It is evident that the two probe-labeling methods, rare earth metal chelate and [γ-^{32}P]ATP, gave similar levels of sensitivity. The limit of sensitivity for the time-resolved fluorescence instrument used in this study is currently 100 amol, with a signal at least 1.5 times that of background.

An additional interesting observation was that the level of amplification did not decrease with increasing concentrations of starting HIV-1 RNA target. This is a different result than was previously observed for a 3SR primer pair used to amplify *E. coli* (3), as well as a different result than has been observed for PCR (9).

Detection of HIV-1 in plasma by culture and 3SR. The results of 3SR and viral plasma culture from plasma samples collected from 19 high-risk pediatric patients less than 5 years of age are as follows: Three patients were positive by both viral culture and 3SR, 11 patients were negative by both methods, 4 patients were positive by 3SR and negative by culture, 1 patient was positive by culture and negative by 3SR but subsequently became positive by 3SR at next testing (within 1 month). All patients who tested positive by 3SR

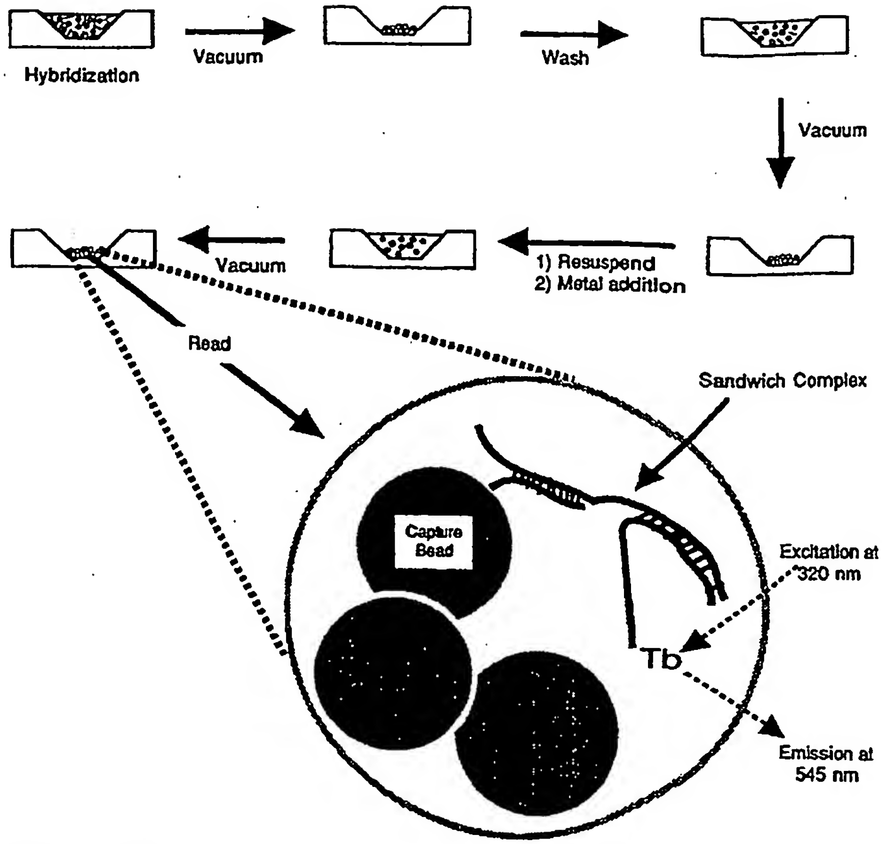

FIG. 2. The bead-based sandwich time-resolved fluorescence assay for the detection of HIV-1. In the time-resolved fluorescence hybridization and detection assay, the 3SR-amplified target sequence is mixed with target-specific capture beads and target-specific probe. The sandwich complex, composed of target, capture beads, and Tb^{3+}-labeled probe, is washed and resuspended and terbium is added. After incubation with the terbium solution, the beads are read in a time-resolved fluorometer at the excitation wavelength of 320 nm and the emission wavelength of 545 nm.

were additionally positive by culture and HIV DNA positive by PCR assay of PBMCs (data not shown).

DISCUSSION

Diseases caused by HIV are rapidly becoming a major cause of early childhood morbidity and mortality (2). More than 50% of infants who acquire the virus vertically show symptoms by the age of 1 year, and approximately 80% show signs by the age of 2 years (21). The period between infection and the development of symptoms is often much shorter in young children than adults; however, the exact period is unpredictable. As more therapeutic choices become available, the need for evaluation of disease will become increasingly important.

In this study, we describe technology which combines the self-sustained sequence replication reaction and time-resolved fluorescence for the detection of HIV-1 RNA in plasma from high-risk pediatric patients. The 3SR technique, unlike PCR, can directly amplify RNA from a sample in an isothermal fashion and produce very high levels of amplification of the specific-single-stranded target in 90 min or less. We report here a 3SR sensitivity of less than 12 HIV-1 RNA copies in 90 min with an amplification level of $>10^{10}$-fold.

We have combined this powerful amplification technique with a detection system that employs polystyrene bead capture of amplified target with a rare earth metal chelate-labeled oligonucleotide probe and time-resolved fluorescence detection. We have compared this technology with plasma viral culture for the rapid and sensitive detection of the HIV-1 virus. Results show that this technology is at least as sensitive and significantly faster than plasma culture for the detection of HIV-1 RNA in plasma.

The rare earth metal chelate used as a label in this assay has several unique properties which make the time-resolved technology suitable as a direct probe-based detection system. The chelate-labeled probes are stable for more than 2 years in a lyophilized state at −20°C; exhibit a large Stoke shift, exciting in the 320-nm range and emiting in the 500- to 600-nm range; and have a life span in the millisecond range, longer than sample autofluorescence, and the individual sample reading time is very fast (22).

The ultimate use of this technology is not limited to detection of HIV infection in pediatric patients (which can also be done very effectively by PCR on PBMCs), but evaluation of the extent and progression of disease. HIV RNA rather than DNA was chosen to be detected because the RNA form of HIV is especially associated with viral

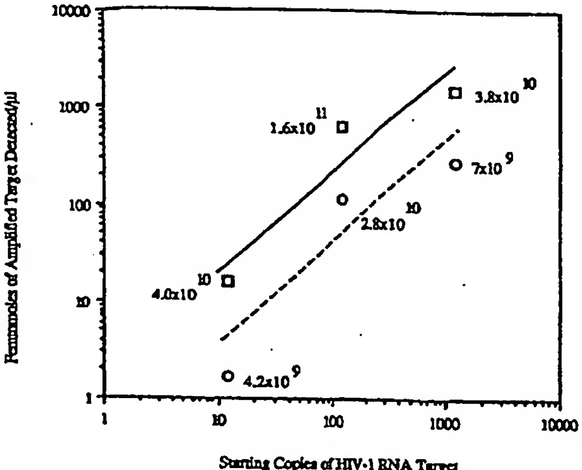

FIG. 3. Sensitivity of the 3SR procedure. 3SR amplification was performed on different amounts of purified HIV-1 RNA. Amplified product was detected by using a bead-based sandwich assay and time-resolved fluorescent (O)- or ^{32}P (□)-labeled probes. Femtomoles of target detected was determined by dividing the time-resolved counts corrected for background by the specific activity of the probe. Amplification levels were determined by dividing the time-resolved counts per microliter by the specific activity of the probe. This value was then divided by the femtomoles of target being amplified in the 3SR reaction.

activity and replication. The choice of plasma is especially appropriate for regularly monitoring pediatric populations, because HIV RNA in plasma correlates with disease progression in adults (5) and because of the limited sample size that is available from neonates and infants. Additionally, zidovudine treatment decreases the amount of virus in plasma, but not PBMCs, from adults (13).

Improved assessment of HIV-1 infection will become increasingly important as antiretroviral therapy is further developed and a better understanding of the events related to the infection of the infant emerge. 3SR amplification coupled with time-resolved fluorescence is a promising technology for investigating the relationship between the presence of HIV-1 RNA in plasma and the progression of disease in HIV-infected pediatric patients because it is at least as sensitive as plasma culture, can be performed in hours instead of weeks, and is potentially automatable. This technology should be important in the assessment of HIV infection, evaluating drug therapies, and understanding the pathogenesis and transmission of HIV-1.

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Design and Evaluation of a Human Immunodeficiency Virus Type 1 RNA Assay Using Nucleic Acid Sequence-Based Amplification Technology Able To Quantify Both Group M and O Viruses by Using the Long Terminal Repeat as Target

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Currently available human immunodeficiency virus type 1 (HIV-1) RNA quantification assays can detect most viruses of the group M subtypes, but a substantial number are missed or not quantified reliably. Viruses of HIV-1 group O cannot be detected by any commercially available assay. We developed and evaluated a quantitative assay based on nucleic acid sequence-based amplification (NASBA) technology, with primers and probes located in the conserved long terminal repeat (LTR) region of the HIV-1 genome. In 68 of 72 serum samples from individuals infected with HIV-1 subtypes A to H of group M, viruses could be detected and quantified. In serum samples from two patients infected with HIV-1 group O viruses, these viruses as well could be detected and quantified. In contrast, the currently used *gag*-based assay underestimated the presence of subtype A viruses and could not detect subtype G and group O viruses. The discrepancy between the results of the two assays may be explained by the number of mismatches found within and among the probe and primer regions of the subtype isolates. These data indicate that LTR-based assays, including the NASBA format chosen here, are better suited to monitoring HIV-1 therapy than are *gag*-based assays in an era in which multiple HIV-1 subtypes and groups are spreading worldwide.

The human immunodeficiency virus type 1 (HIV-1) RNA level in plasma or serum has become one of the most important markers for monitoring HIV-1-infected patients. Other than HIV-1 DNA, it is the only evidence for mother-to-child transmission, since maternal antibodies present in infant serum hamper antibody-screening assays. The HIV-1 RNA level is the most valuable marker for predicting disease progression in nontreated patients (10, 13, 21, 22, 34) and is highly useful for evaluating the effectiveness of antiretroviral drug therapy (14, 26, 43). The decision to start antiretroviral drug therapy is currently made on the basis of the viral RNA level (31). A patient with an HIV-1 RNA level of less than 10,000 copies per ml generally will not progress to AIDS for at least 5 years (22, 34). Highly active antiretroviral therapy, consisting of treatment with a combination of three drugs, results in a decline of the viral RNA level of approximately 99% (28). The therapy's goal, for optimal delay of disease, is to decrease the viral RNA level until it cannot be detected by RNA quantification assays.

Although HIV-1 subtype B has been the predominant cause of AIDS in Europe and the United States, other HIV-1 subtypes, particularly subtypes A and C, are now taking over. As these different clades of HIV-1 spread rapidly around the world, there is an increased need for assays that can reliably quantify the level of RNA of all known subtypes, i.e., group M subtypes A to H and group O viruses, in plasma, serum, or culture supernatants.

Commercially available RNA quantification assays are based either on the amplification of a fragment of the *gag* gene of the HIV-1 genome (e.g., NucliSens HIV-1 QT assay [Organon Teknika, Boxtel, The Netherlands] or Amplicor version 1.5 HIV Monitor test [Roche Diagnostics, Basel, Switzerland]) (15, 23, 24, 41, 42) or on the direct detection of HIV-1 RNA by hybridization with labeled probes (e.g., Quantiplex HIV 3.0 assay; Chiron Diagnostics, Emeryville, Calif.) (27, 35, 36, 46). The NucliSens and Amplicor assays were developed with reagents derived from HIV-1 subtype B, but they can detect most group M viruses. The Quantiplex assay uses 45 target probes designed to hybridize with all known HIV-1 group M viruses and is thus more likely than the other two assays to detect and quantify genetically divergent HIV-1 subtypes (7, 12, 25). It has been reported that there is no difference in general performance, for instance, with regard to sensitivity, accuracy, and reproducibility, among the three assays (7, 11, 30, 32, 37, 38) even though some viruses not detected by one assay have been detected by another assay (1, 8, 25). None of the assays can detect HIV-1 group O viruses (12, 20, 29). The sensitivity of both the Quantiplex and the NucliSens assays, if an ultrasensitive protocol is applied, is currently 50 copies of RNA per ml of plasma or serum (5, 44), with an input of 1,000 or 200 μl, respectively. The Amplicor assay has variable sensitivity, with a detection limit of generally ranging from 30 to 60 RNA copies per ml of plasma when an ultrasensitive protocol is applied with an input of 500 μl.

We developed and evaluated a new, broad-clade HIV-1 RNA quantification assay based on nucleic acid sequence-based amplification (NASBA) technology. The evaluation was performed with plasma or serum samples that together contained all group M subtypes and group O viruses. We show that the number of mismatches in sequences of primers and probes

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was the major determinant of accuracy in the detection and quantification of HIV-1 RNA.

MATERIALS AND METHODS

Three-calibrator *gag*-based NASBA and one-calibrator long terminal repeat (LTR)-based NASBA. The three-calibrator *gag*-based NASBA is a commercially available assay (NucliSens HIV-1 QT assay; Organon Teknika). The assay was performed by following the instructions of the manufacturer.

Four regions having highly conserved sequences, which were found in the 5' end of the genomic RNA (LTR region) after screening of the known HIV-1 genomes (17), were used to develop an LTR-based NASBA. The assay was based on standard NASBA technology (42) but used one internal calibrator (Q) molecule instead of three, as in the *gag*-based assay (15, 41, 42). A fragment of approximately 135 bases of antisense RNA was amplified and detected with two primers and two probes. Calibrator molecules were added to a 200-μl plasma or serum sample, and RNA was isolated by a silica-based method (4). Five microliter volumes of the 50 μl of isolated viral RNA and calibrator RNA were used in the NASBA reaction with a 5' sense primer (5' CTCAATAAAGCTTGCCTTGA) (HIVHXB2CG [GenBank accession no. K03455] nucleotides [nt] 508 to 523) and a 3' antisense primer elongated with a T7 sequence (in lowercase italics) (5' *aattctaatacgactcactatagggagag*GGCGCCACTGCTAGAGA) (nt 643 to 628) were used to amplify the LTR fragment. After 1.5 h of incubation at 41°C, 5 μl of the reaction mixture was diluted 31 times in detection diluent (Organon Teknika). From the diluted sample, 5 μl was added either to a mixture of ruthenium tag-labeled wild-type detection (5' AATGTGTGCCCGTCTGTT) (nt 555 to 572) and biotin-labeled capture (5' TCTGGTAACTAGAGATCCCTC) (nt 580 to 600) probes or to a mixture of Q detection and identical capture probes. The detection probes were labeled so they could be detected by electrochemiluminescence (3). The number of wild-type RNA copies per milliliter of serum was calculated by the ratio between the wild-type signal and the Q signal. Serum samples in which no viral RNA could be detected were reanalyzed by following an ultrasensitive protocol (44).

Ultrasensitive protocol for NASBA. An ultrasensitive protocol for NASBA (UltraSens protocol) (44), which improved the sensitivity of both the *gag*-based and the LTR-based NASBAs to 50 copies/ml, had already been developed. Briefly, the eluted nucleic acids remaining from the isolation procedure described in the previous section were taken off of the silica beads. The noneluted nucleic acids, still attached to the silica beads, were eluted again in 70 μl of elution buffer (Organon Teknika) and pooled with the previously eluted nucleic acids. The nucleic acids were precipitated with Pellet Paint (Novagen, Madison, Wis.) and ethanol. After the pellet was washed, the nucleic acids were again amplified and detected by following the standard NASBA amplification and electrochemiluminescence detection procedure.

Samples. Seventy-two serum samples taken from individuals suspected or known to be infected with a non-B HIV-1 subtype were selected from the collection at the outpatient clinic of the Academic Medical Center, Amsterdam, The Netherlands. Most of these individuals were non-European and non-U.S. immigrants to The Netherlands, who probably were infected in their home country, or were individuals known or suspected to be infected with an HIV-1 strain of non-European and non-U.S. origin. They had been identified by a thorough epidemiological investigation that is part of the routine evaluation of every newly diagnosed HIV case at the outpatient clinic of the Academic Medical Center. In addition, four serum samples from two individuals infected with an HIV-1 group O virus (ANT70 and partner [9]), taken before and during antiretroviral therapy, were the kind gift of G. van der Groen and W. Janssens (Institute for Tropical Medicine, Antwerp, Belgium). Sequence analysis was performed on the *gag* sequences of all samples to identify the viral subtypes.

To obtain supernatants from the viral cultures of subtypes A to G, infectious virus stocks were collected and prepared by the World Health Organization (WHO) Network for HIV Isolation and Characterization (18). Expanded virus stocks were produced (45) by the inoculation of 4.0×10^6 phytohemagglutinin-stimulated donor peripheral blood mononuclear cells with a supernatant from cultures of the primary isolate. After being incubated and washed, cells were resuspended in culture medium and incubated at 37°C. Cell-free supernatant was harvested after 10 to 11 days.

PCR. Nucleic acids were isolated from 200 μl of serum by a silica-based method (4). After washing and elution from the silica with 100 μl of sterile water, 10 μl of the eluate was used in a reverse transcription reaction with avian myeloblastosis virus reverse transcriptase. For amplification of the *gag* gene, we used the antisense primer 3' SK39 (5' GCATTCTGGACATAAGACAAGGACCAAA) (nt 1658 to 1631). For amplification of the 5' LTR, we used 3' L-GagUniM2 (5' GCACCCATCTCTCTCCTTCTAGCCTCCGC) (nt 797 to 759). After incubation of the eluate for 45 min at 41°C, a PCR mixture containing the sense primers 5' Gag-1 (GCGAGAGCGTCAGTATTAAGC) (nt 796 to 816) for the *gag* gene and 5' L-R1-M2 (5' GGTCTCTCTTGTTRGACCAGATYTGAGCC) (nt 455 to 484) for the 5' LTR, PCR buffer, deoxynucleoside triphosphates, 2.5 mM $MgCl_2$ for the *gag* gene and 5 mM $MgCl_2$ for the 5' LTR, and 2 U of *Taq* polymerase was added. After incubation for 5 min at 95°C, the reaction mixture was subjected to 35 cycles of amplification (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C). Nested PCRs with 25 cycles of amplification each were performed before direct sequencing. The nested *gag* gene product was obtained after amplification with the primers 5' Gag-2-SP6 (sense; 5' *atttaggtgacactatag*GGGAAAAATTCGGTTAAGGCC) (nt 836 to 857) and 3' Gag AE3-T7 (antisense; 5' *taatacgactcactataggg*TAGGACCCTAATTTATTTTATCA) (nt 1610 to 1588; SP6 and T7 sites are in lowercase italics). The product of the nested 5'-LTR PCR was obtained after amplification with the primers 5' L-T7-R2M2 (sense; 5' *taatacgactcactataggg*GAGCCTGGGAGCTCTCTGGCTA) (nt 479 to 500) and 3' L-GagM-SP6 (antisense; 5' *atttaggtgacactatag*AGCAAGCCGAGTCCTGCGTC) (nt 707 to 688). The conditions for these PCRs were similar to those described for the first PCR but with a concentration of 4 mM instead of 5 mM $MgCl_2$ for the nested 5'-LTR PCR. The presence of amplified PCR products was verified with 1% agarose gels stained with ethidium bromide.

DNA sequencing. Both strands of the nested-PCR fragments were directly sequenced with the SP6 and T7 primer sequences. Sequencing was performed with *Taq* dye primers (Applied Biosystems, Foster City, Calif.) and the Thermo Sequenase fluorescence-labeled primer cycle-sequencing kit (Amersham International, Little Chalfont, England). The sequence products were analyzed on an automatic DNA sequencer (model 373A stretch; Applied Biosystems).

The sequences were aligned manually. Phylogenetic analysis of the *gag* gene sequences of all serum samples was performed by the neighbor-joining method of the TREECON program (39). The distance matrix was generated by Kimura's two-parameter estimation (16).

Statistical analysis. Statistical analysis was performed by using the Pearson product moment correlation procedure as well as the paired *t* test as implemented in the SigmaStat version 1.0 software package (Jandel Corporation, San Rafael, Calif.).

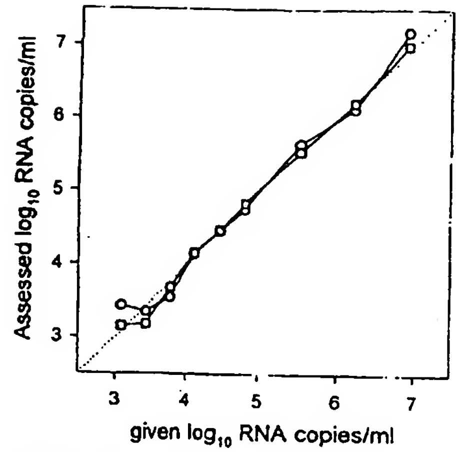

FIG. 1. Assessed viral RNA levels versus given viral RNA levels in serial dilutions of HIV-1 HXB3 (19) in plasma as determined with the LTR-based (O) and the *gag*-based (□) NASBAs. The results shown are the means of at least three independently performed experiments.

RESULTS

Comparison of the quantitative performance of the *gag*- and LTR-based NASBAs on HIV-1 subtype B RNA. The *gag*-based NASBA, based on subtype B sequences, has already shown its ability to detect and quantify HIV-1 subtype B genomic RNA (13, 38, 40–42, 47). We tested whether the new LTR-based NASBA could equal its performance. The two assays were compared with a panel consisting of dilution series of a well-characterized subtype B standard (HXB3) in 0.2 ml of human plasma (19). Analysis of the dilution series yielded similar results for both assays (Fig. 1). The quantification of both assays was linear and accurate over a range of 10^3 to 10^7 copies of genomic HIV RNA per ml when a sample volume of 0.2 ml was applied. The precision and accuracy of the LTR-based NASBA were within 0.2 and 0.1 $\log_{10}$, respectively, for up to 250 copies of genomic HIV RNA per input volume (0.2 ml). This result was determined with a group of 47 human plasma samples mixed with known amounts of HXB3 (19) (data not shown). The analytical sensitivity of the assay, in which ampli-

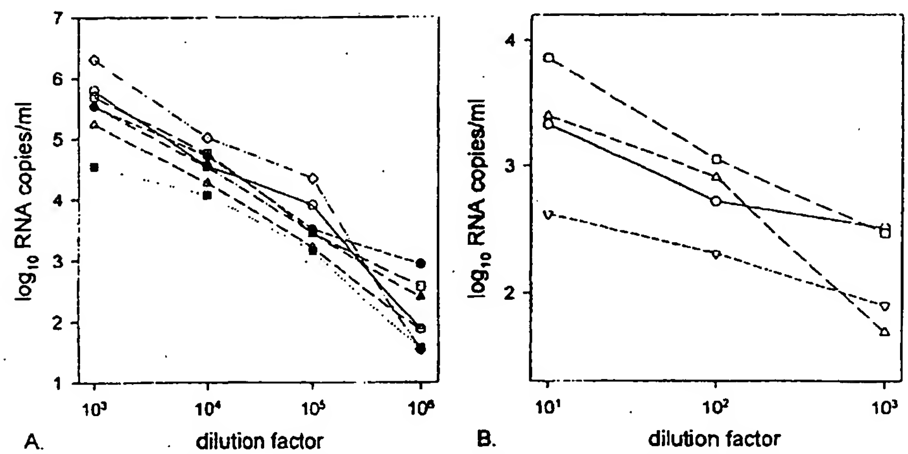

FIG. 2. Assessed viral RNA levels versus dilution factor of supernatant from cultures of HIV-1 group M subtypes A (O), B (□), C (△), D (●), E (■), F (▲), and G (◇) (A) and from four different HIV-1 group O viruses (B).

fication occurred in 50% of the reactions, was approximately 10 genomic RNA copies per reaction. An input volume of 0.2 ml led to a sensitivity of 500 copies of genomic HIV RNA per ml, as only 1/10 of the sample was used in a reaction. The sensitivity was improved to 50 copies of genomic RNA per ml when the UltraSens protocol was applied.

Linear quantification capacities of the LTR-based NASBA on various HIV-1 subtypes. The linear quantification abilities of the LTR-based assay were determined by serial dilutions of a viral culture supernatant for each subtype (A to G) of the HIV-1 M group and for four viruses of the HIV-1 O group. The results of representative dilution series for the HIV-1 M and O groups are plotted in Fig. 2A and 3B, respectively. For subtypes A to G of the HIV-1 M group, a linear decrease of the assessed viral RNA levels as the level of dilution increased was observed, indicating that all subtypes were quantified similarly. The variation in initial RNA levels was determined by differences in RNA input. One representative experiment of three that were performed for each subtype was plotted (Fig. 2). The slope of the linear decrease of the viral RNA levels was not as steep for the group O viruses. This finding suggests that the efficiency of quantification was less for the group O viruses than for the group M viruses and was probably due to a greater number of mismatches in the capture probe.

RNA quantification in serum samples from 72 individuals infected with HIV-1 group M viruses of subtypes A to H and from two individuals (four samples) infected with HIV-1 group O viruses. Viral subtypes of the viruses were determined based on phylogenetic analysis of the sequences of the *gag* genes. The serum samples with a viral RNA level below the detection limit

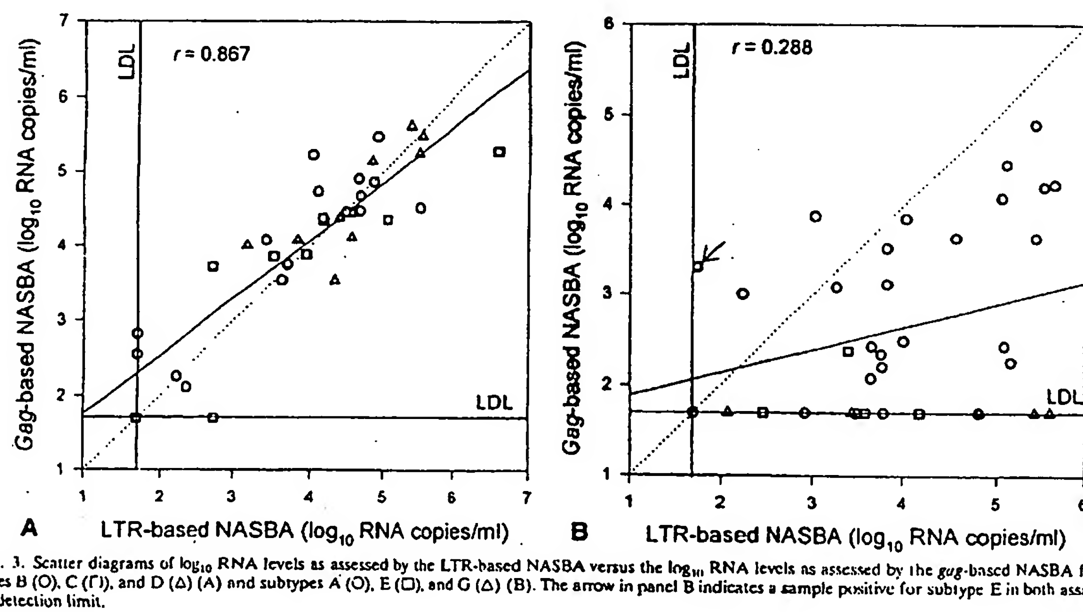

FIG. 3. Scatter diagrams of $\log_{10}$ RNA levels as assessed by the LTR-based NASBA versus the $\log_{10}$ RNA levels as assessed by the *gag*-based NASBA for HIV-1 subtypes B (O), C (□), and D (△) (A) and subtypes A (O), E (□), and G (△) (B). The arrow in panel B indicates a sample positive for subtype E in both assays. LDL, lower detection limit.

of either the LTR- or the *gag*-based assay were reexamined by using the UltraSens protocol, which has a lower detection limit (50 copies/ml). The *gag*-based NASBA was unable to detect viral RNA in samples containing subtype G or HIV-1 group O viruses, whereas the LTR-based assay could detect the RNA of all tested subtypes or groups. For subtype A viruses, RNA levels were significantly lower ($P < 0.0001$) with assessment by the *gag*-based NASBA than they were with assessment by the LTR-based assay. For all samples, the RNA levels for subtype E viruses as determined by the *gag*-based assay were lower than as determined by the LTR-based assay, but not significantly ($P = 0.26$).

To facilitate analysis, the serum samples were divided into two groups. The first group contained subtypes B, C, and D (Fig. 3A), whereas the second group contained subtypes A, E, and G (Fig. 3B). Only one serum sample each was available for subtypes F and H, and for group O, only four serum samples from two patients were available. Results for these six serum samples were therefore not plotted in the diagrams. The correlation coefficient (r) for the *gag*-based and LTR-based NASBA results for all sera together, including those not plotted, was 0.52 ($n = 76$; $P < 0.0001$). For the group containing the subtype B, C, and D viruses, a strong correlation could be found between the two assays ($r = 0.87$; $P < 0.0001$), but this correlation was not as strong for the group with subtype A, E, and G viruses ($r = 0.29$; $P = 0.089$). In total, 16 viruses of various subtypes of the M group (and the four O-group viruses) could not be detected by the *gag*-based NASBA. Of these, five were subtype A, one was subtype C, one was subtype D, two were subtype E, six were subtype G, and the remaining one was the only subtype H in our serum sample set; therefore, 13 of these 16 serum samples (81%) were in the group containing A, E, and G subtypes.

The LTR-based NASBA was unable to detect viral RNA in four serum samples. Two of these, one subtype A and one subtype D, were negative for viral RNA by both assays. The other two serum samples contained subtype B viruses, which could be detected by the *gag*-based NASBA only after the UltraSens protocol was applied. This result could indicate that for a limited number of subtype B isolates the detection limit of the *gag*-based NASBA is lower than that of the LTR-based NASBA. Finally, one serum sample (Fig. 3B) contained a subtype E virus and was positive by both assays; however, it was positive in the LTR-based NASBA only with the UltraSens protocol.

Analysis of mismatches in primer and probe regions. To explain the discrepancies in assessed viral RNA levels between the two assays, we sequenced the relevant LTR and *gag* regions and analyzed the number of mismatches for the primers and the probes. For analysis, insertions and deletions present only in the noncoding LTR region were counted as one mismatch each. For all samples, the number of mismatches for the primers and probes counted for the *gag*-based NASBA (range, 0 to 17; $n = 71$; mean, 7.85; median, 7) was significantly higher ($P < 0.0001$) than that counted for the LTR-based NASBA (range, 0 to 5; $n = 64$; mean, 0.61; median, 0). The means and standard deviations of the viral RNA levels ($\log_{10}$ copies per milliliter) for all samples per subtype, as assessed by the LTR-based and the *gag*-based NASBAs, are plotted in Fig. 4A. The means and standard deviations of the mismatches per subtype for the primers and probes of the LTR- and *gag*-based NASBAs are plotted in Fig. 4B. We found a significant inverse correlation between the number of mismatches and viral RNA levels detected for the *gag*-based NASBA ($r = -0.78$; $P = 0.023$) which was not present for the LTR-based NASBA. This finding implied that complete or partial assay failure was due to primer

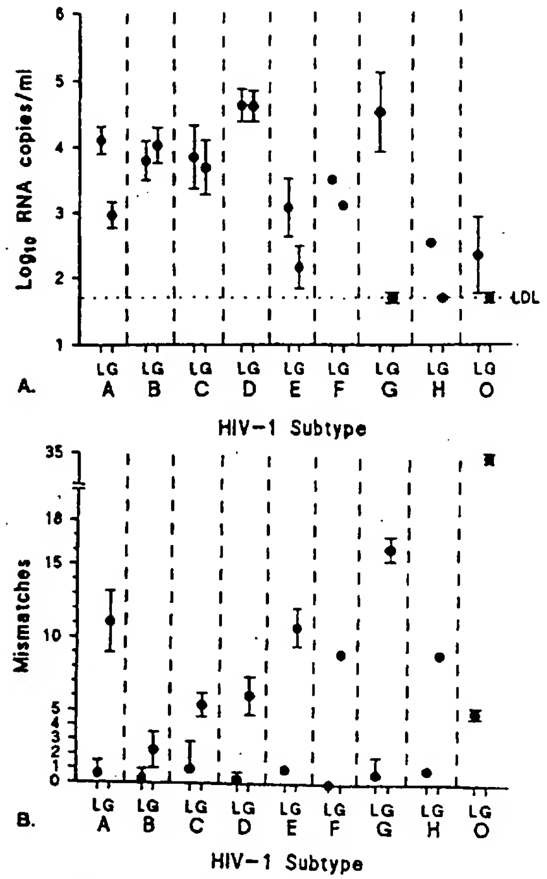

FIG. 4. (A) Mean $\log_{10}$ RNA level with standard deviation (error bars) for each HIV-1 subtype as assessed by the LTR-based NASBA (L) and the *gag*-based NASBA (G). LDL, lower detection limit. (B) Mean number of mismatches with standard deviation (error bars) for each subtype for the primers and probes of the LTR-based assay (L) and the *gag*-based assay (G).

and probe mismatches. The mismatches resulted in assessments of significantly lower or absent viral RNA levels for subtype A and G and group O viruses by the *gag*-based assay. The difference in viral RNA level in the previously described subtype E serum sample, as estimated by the two assays, was not explainable by the number of mismatches for primers and probes for each assay.

DISCUSSION

Because various subtypes of HIV-1 are rapidly spreading around the world, HIV-1 RNA quantification assays that can detect all known subtypes of HIV-1 group M and group O viruses are required. We have developed a new NASBA-based assay that uses the conserved LTR region at the 5′ end of the genomic RNA. We have shown that the LTR-based assay is as good in standard subtype B quantification as the existing *gag*-based assay (NucliSens HIV-1 QT assay; Organon Teknika). The lower detection limits were similar for both assays. By comparing dilution series of a panel of group M subtype isolates from the WHO collection, we have shown that the LTR-based assay quantifies these viruses as efficiently as it does subtype B viruses. In contrast, the group O viruses were less

efficiently quantified than group M viruses (i.e., the viral load was underestimated), probably due to mismatches in the capture probe. Adaptation of the capture probe to a group O matching sequence would most likely resolve this matter. By using serum samples for which the viral subtype was determined by phylogenetic analysis of the *gag* gene, we have shown that the two assays are similar in their ability to quantify subtypes B to F of HIV-1 group M. However, the LTR-based assay is better suited to quantify subtypes A and G of group M and the group O viruses, as well as the only group M, subtype H, virus in our serum sample set. The set also included some proven recombinant viruses (6), which were also detected and quantified by the LTR-based assay.

The most important improvement in the LTR-based NASBA, compared to the *gag*-based NASBA, is its decreased number of mismatches for primers and probes. We found a strong inverse correlation ($r = -0.78$; $P = 0.023$) between calculated viral RNA levels and the number of mismatches found for the *gag*-based NASBA. It can therefore be concluded that for subtype A and G viruses, as well as for group O viruses, the RNA levels as assessed by the *gag*-based NASBA will be underestimated or absent. For these viruses, the RNA levels will be detected with more efficiency and accuracy by the LTR-based NASBA.

It has been reported that the three most widely used commercially available assays, namely, NucliSens HIV-1 RNA, Amplicor HIV-1 Monitor, and Quantiplex HIV-1 RNA, are similar in sensitivity, accuracy, and reproducibility (7, 11, 30, 32, 37, 38) but that the Quantiplex HIV-1 RNA assay is slightly more effective for quantification of isolates of certain subtypes (7). Like the *gag*-based NucliSens assay, the *gag*-based Amplicor assay underestimates or cannot detect subtype G viruses (2, 8). Neither the *gag*-based NASBA, the Amplicor assay, nor the Quantiplex assay was able to detect and quantify group O viruses (12, 20, 29), but the LTR-based NASBA could.

A new group of HIV-1 viruses, the N group, was recently reported (33). This group is genetically different from groups M and O. Using a published genomic sequence (GenBank accession no. AJ006022) of a member (YBF30) of this group and analyzing the number of mismatches, we could speculate whether this virus might be detected with either the *gag*-based or LTR-based NASBA. Since 22 mismatches were present for the *gag*-based NASBA primers and probes and this number lies between those for subtype G and group O viruses, which are not detected, it is unlikely that the new group can be detected by the *gag*-based NASBA. With the LTR-based NASBA, however, only four mismatches were found. This is less than was found for group O viruses, so it seems likely that this assay can detect and quantify the new group N viruses, provided that their LTR sequence resembles that of their representative member, YBF30.

Our LTR-based NASBA would be of use for testing infants born of HIV-1-positive mothers. These infants cannot be diagnosed HIV-1 positive based on the presence of antibodies against HIV-1 antigens, because maternal HIV-1 antibodies are present in the serum of these infants. The presence of HIV-1 DNA or RNA must be detected directly in cellular material or serum from the infant to make the diagnosis. Improving the chance of detection by changing the amplification region from *gag* to the LTR will probably lead to prompt diagnosis of HIV-1 in infected infants, especially if the infants are infected with a subtype A or G or a group O virus. An additional advantage of the ultrasensitive NASBA format over the Quantiplex and the ultrasensitive Amplicor assay format is the smaller serum volume (200 versus 1,000 versus 500 μl, respectively) necessary to detect HIV-1 RNA with similar sensitivity.

Another application of the LTR-based NASBA could be the monitoring of patients receiving antiviral therapy. Decreased efficiency and accuracy in assessments of viral RNA levels could impact not only the start of therapy but also the judgment of treatment failure or success. Often, the decision to start highly active antiretroviral therapy is made on the basis of the viral RNA levels (19). If assay failure leads to a too-low estimation of viral RNA levels, such therapy could be delayed or omitted, putting the infected individual at an increased risk for developing AIDS (21, 22). If the *gag*-based NASBA is used to monitor the treatment of individuals infected with group M, subtype A or G, viruses or group O viruses, the viral RNA levels could be determined, too early, to be below the lower detection limit, falsely indicating therapy success. The viral RNA level will rise above the lower detection limit more slowly, causing an unnecessarily later switch to a new drug regimen when drug resistance does begin to appear.

In summary, compared with the *gag*-based NASBA, our LTR-based NASBA has improved capacities for quantification of the HIV-1 group M, subtype A, virus as well as for the detection and quantification of the subtype G and group O viruses. This assay is a major advancement in HIV diagnostics, affecting decision management for the start and monitoring of therapy and the diagnosis of HIV-1-infected infants.

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P.D. 16-07-1998

p. 1 = (1)

ID AAV19509 standard; DNA; 22 BP.
XX
AC AAV19509; XP-002226198
XX
DT 16-JUL-1998 (first entry)
XX
DE Retroviral DNA base sequences amplifying primer OG24.
XX
KW Retrovirus; AIDS; serum; HIV; human immunodeficiency virus;
KW antigen measurement; diagnosis; nested PCR primer; ss.
XX
OS Synthetic.
OS Human immunodeficiency virus type 2.
XX
PN JP10094394-A.
XX
PD 14-APR-1998.
XX
PF 20-SEP-1996; 96JP-0271467.
XX
PR 20-SEP-1996; 96JP-0271467.
XX
PA (EIKE) EIKEN KAGAKU KK.
XX
DR WPI; 1998-279230/25.
XX
PT Retrovirus reacting with AIDS patient serum - useful for the exact
PT diagnosis of an unknown AIDS causing virus
XX
PS Examples; Page 7; 16pp; Japanese.
XX
CC This primer is used in the nested PCR amplification of the DNA base
CC sequences isolated from a retrovirus particle collected from the blood
CC of an AIDS patient. The specification provides DNA base sequences
CC encoding a retroviral protein which reacts with serum of AIDS patients.
CC It provides an antigen for the detection of an antibody against
CC retrovirus which consists of a peptide derived from these base sequences.
CC The invention provides a method for antigen measurement in which the
CC above antigen is contacted with a sample blood to determine
CC immunoglobulin reacting with the antigen and a method for screening the
CC infection of retrovirus other than HIV-1, HIV-2 subtype A which can be
CC collected from an AIDS patient blood by the above antibody measurement.
CC The method can diagnose exactly an unknown AIDS-causing virus.
XX
SQ Sequence 22 BP; 7 A; 5 C; 7 G; 3 T; 0 other;
aagggtccta acagaccagg gt 22
//

```
ID   AAT06604 standard; DNA; 18 BP.
XX
AC   AAT06604;
XX
DT   21-JUN-1996  (first entry)
XX
DE   LTR outer reverse (776) primer for SIV amplification.
XX
KW   primer; PCR; polymerase chain reaction; simian immunodeficiency virus;
KW   SIV; non-infectious; viral particle; immunity; vaccine; diagnosis;
KW   assay; ss.
XX
OS   Synthetic.
XX
PN   US5470572-A.
XX
PD   28-NOV-1995.
XX
PF   16-JUL-1993;   93US-0093336.
XX
PR   16-JUL-1993;   93US-0093336.
XX
PA   (UYPU-) UNIV PUERTO RICO.
XX
PI   Kraiselburd E;
XX
DR   WPI; 1996-019831/02.
XX
PT   New immunogenic non-infections SIV viral particles  - useful as a
PT   vaccine, for diagnostic purposes and for antibody production
XX
PS   Example 4; Column 10; 15pp; English.
XX
CC   Simian immunodeficiency virus (SIV)-specific nucleotide sequences in
CC   genomic DNA obtained from cell cultures were amplified by PCR, using
CC   oligonucleotide primers derived from the SIV-SM-H4 long terminal repeat
CC   (LTR) genome region.  The nucleotide sequences are shown in AAT06603-06.
CC   The position of the 5' nucleotide in the complete SIV-SM-H4 sequence is
CC   given in parantheses above. Non infectious SIV viral particles may be
CC   used to immunise a subject or to generate polyclonal or monoclonal
CC   antibodies for use in diagnostic assays.
XX
SQ   Sequence 18 BP; 5 A; 5 C; 6 G; 2 T; 0 other;
     gggtcctaac agaccagg                                            18
//
```

XP-002226197

P.D. 21-06-1996

p. 1 = 1

XP-001006264

Identification of mixed HIV-1/HIV-2 infections in Brazil by polymerase chain reaction

pd 00-00-1991

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p. 1293 - 1299 (7)

Analysis of sera from hospitalized Brazilian patients by whole-virus lysate-based enzyme immunoassay and Western blot indicated that 0.4% were reactive to HIV-2 alone while 4% were reactive to both HIV-1 and HIV-2. When these sera were tested for HIV antibody by type-specific peptide enzyme immunoassays, dual seropositivity was confirmed in only 0.4% of patients. To define genetically the HIV strains within the population, we analyzed peripheral blood mononuclear cells from selected seropositive patients for the presence of HIV-1 and HIV-2 proviral DNA using the polymerase chain reaction (PCR). Independent primers/probes sets were used for the amplification and detection of viral sequences from the long terminal repeat (LTR), gag, and protease (*prt*) gene regions. Our findings confirmed the serologic evidence of HIV-2 in Brazil and determined the extent of mixed HIV-1 and HIV-2 infections. Detailed evaluation of the amplified viral protease sequences by endonuclease restriction analysis and DNA sequencing independently confirmed mixed HIV-1 and HIV-2 infections in the two patients seropositive for HIV-1 and HIV-2. The data further indicated that these isolates are distinct from the HIV laboratory standards. We interpret the combination of culture and PCR findings to demonstrate the presence of both HIV-1 and HIV-2 in Brazil.

AIDS 1991, 5:1293–1299

Keywords: Polymerase chain reaction, HIV-1, HIV-2, Brazil, protease.

[For editorial comment, see pp 1379–1380]

Introduction

Collaborative studies were initiated by the Brazilian Ministry of Health and the US Centers for Disease Control (CDC) in 1989 to develop a clinical case definition of AIDS in Brazil. A serosurvey was conducted of patients in two hospitals that served as referral centers for individuals with AIDS within a major metropolitan area. Analysis of the sera suggested the presence of HIV-1, HIV-2, and potential mixed HIV-1 and HIV-2 infections, based on both HIV-1- and HIV-2-seropositive hospitalized individuals (data not shown). The value obtained for dually reactive sera was comparable to the 3% estimate for mixed HIV infections among Brazilian individuals with known high-risk practices, previously reported by Cortes *et al.* [1]. Although these serologic findings are consistent with the presence of HIV-2 in Brazil because of the cross-reactivity

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Note: Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services.

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of HIV-1 and HIV-2 [2,3], they do not by themselves provide sufficient information on the types of HIV circulating within the population and the prevalence of true mixed HIV infections.

The initial seroprevalence surveys involved whole-virus lysate-based enzyme-linked immunoassays (VEIA; Genetic Systems, Seattle, Washington, USA). Serologic cross-reactivity to HIV-1 and HIV-2 within such assays has been reported to range from 50 to 91% [2,3]. This level of cross-reactivity is significant in light of the rising number of individuals possessing antibodies to the Gag, Pol, and Env proteins of both HIV-1 and HIV-2 [4–7]. It remains unclear whether this dual seropositivity is due to: (1) a broad immune response against infection with a single HIV serotype; (2) infection with a unique third virus containing epitopes common to both serotypes; (3) a mixed HIV infection.

To define the HIV status within the Brazilian study subjects, we used the polymerase chain reaction (PCR) to analyze peripheral blood mononuclear cells (PBMC) from selected seropositive patients and seronegative study controls for HIV-1 and HIV-2 proviral DNA.

PCR analysis has been used successfully to determine genetically the HIV serotype(s) within patients in the absence of definitive serologic findings [8]. In the present study, amplified sequences from the viral long terminal repeat (LTR), *gag*, and protease (*prt*) gene regions were evaluated for genotypic markers characteristic of HIV-1 or HIV-2. This approach avoids the difficulties associated with separating two distinct viral subtypes from a common culture in which there is a tendency for the most adaptive strain to dominate in prolonged virologic cultures.

Our data from PCR amplification of HIV-specific sequences, followed by endonuclease cleavage studies and sequence analysis, confirm the presentation of two mixed HIV infections, and the presence of HIV-2 infection in Brazil.

Materials and methods

Enzyme-linked immunoassays

Serum samples were evaluated for HIV-1 and HIV-2 antibodies by VEIA and type-specific enzyme immunoassay (PEIA) to synthetic peptides from the immunodominant domains of the transmembrane proteins of each virus (Genetic Systems). Western blot (WB) analysis was performed as previously described by Tsang *et al.* [9] using whole virus lysates (BioRad, Richmond, California, USA; Genetic Systems). The EIA and Western blot assays were performed and interpreted according to the manufacturer's instructions.

Culture of virus

PBMC from six representative seropositive patients and three seronegative controls were cultured in the presence of phytohemagglutinin (PHA)-stimulated normal donor PBMC and sampled for the detection of proviral DNA at 3-day intervals up to 2 months by PCR. Culture conditions have previously been described [10]. Normal donors for PBMC were seronegative and did not express proviral DNA as determined by PCR. The standard laboratory reference strains for HIV-1 were the pZ6Neo infectious clone of HIV_{Z6} (GenBank, Cambridge, Massachusetts, USA; accession number KO3458) and the Centers for Disease Control (CDC) 855 isolate. For HIV-2, $HIV\text{-}2_{ROD}$ (GenBank; accession number M15390) and CDC 618 strains were used.

Table 1. Primers and probes for HIV amplification by the polymerase chain reaction.

Primer pairs and probes	Sequence (5' to 3')	Locations*
HIV-1		
gag gene region		
SK38 primer	ATAATCCACCTATCCCAGTAGGAGAAAT	1551–1578
SK39 primer	TTTGGTCCTTGTCTTATGTCCAGAATGC	1638–1665
SK19 probe	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC	1595–1635
prt (protease) gene region		
DP16 primer	CCTCAAATCACTCTTTGGCAAC	2256–2277
DP17 primer	AAAATTTAAAGTGCAGCCAAT	2531–2552
DP18 probe	TGCAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAA	2378–2420
HIV-2		
Long terminal repeat		
CO19 primer	GACTGCAGGGACTTTCCAGA	9383–9403
CO20 primer	GAACACCCGGGCTCTACCTG	9555–9574
CO21 probe	GAGGACCTGGTGGGGAACGC	9431–9450
prt gene region		
DP26 primer	CCTCAATTCTCTCTTTGGAAAA	2084–2105
DP27 primer	TAGATTTAATGACATGCCTAA	2360–2380
DP28 probe	ACTCAATACTAGCACGAATAGAGTTAGGGAACAATTATAG	2172–2211

*HIV-1 gag, isolate HIVSF2, GenBank accession number K02007; protease, isolate HIVZ2Z6, GenBank accession no. M22639. HIV-2 isolate $HIV\text{-}2_{ROD}$, GenBank Accession no. M15390.

PCR

The type-specific primers and probes for PCR amplification of proviral DNA are shown in Table 1. For amplification of the HIV-1 *gag* region and viral protease gene, oligonucleotide primer pairs SK38/39 and DP16/17, respectively, were used. Type-specific detection of the HIV-2 LTR region and viral *prt* was based on amplification using CO19/21 and DP26/27 primer sets, respectively. The PCR mixture containing 0.15×10^6 PBMC (corresponding to 1 µg genomic DNA) was processed as described by Saiki *et al.* [11] in an automated temperature device (Perkin Elmer Cetus, Norwalk, Connecticut, USA). After 35 cycles of amplification, 10 µl of the original 100 µl reaction mixture was analyzed for the sequence of interest by oligomer hybridization (OH) using an appropriate ^{32}P-end-labeled oligonucleotide probe. Repeatedly reactive specimens were considered positive, and field isolates from Africa or the United States served as internal controls within the assays to ensure the type-specificity of the reactions.

Restriction enzyme digestion patterns analysis

All endonuclease cleavage reactions were conducted in accordance with the instructions provided by the manufacturers (Gibco/BRL, Life Technologies Inc., Gaithersburg, Maryland, USA; New England Biolabs Inc., Beverly, Massachusetts, USA; Promega Inc., Madison, Wisconsin, USA). PCR diagnostic fragments were generated by *BstNI* cleavage, following *gag* or LTR-specific amplifications, and fragments were resolved on 20% polyacrylamide gels. HIV-1- or HIV-2-specific diagnostic fragments representing viral protease sequences were digested with *AluI* or *HinfI*, respectively. Cleavage fragments were separated by electrophoresis on 20% polyacrylamide gel and visualized by ethidium bromide staining or autoradiography to develop a restriction map of the amplified sequences spanning the viral *prt* gene. Products of three independent amplifications for each sample were analyzed.

Cloning and sequencing

PCR-amplified HIV-1 and HIV-2 protease genes from the Brazilian samples and reference strains were cloned in a bacterial plasmid which carries a resistance marker for ampicillin and multicloning site (pBluescript II SK; Stratagene, La Jolla, California, USA) according to the method of Sambrook *et al.* [12]. These clones provided a stable source of material for endonuclease restriction analysis and subsequent DNA sequencing. Clones derived in this manner are designated with the prefix pMO. The fidelity of the PCR amplification and cloning protocols was confirmed by comparison of reference strain sequences with those previously published. The chain termination sequencing method, as described by Tabor and Richardson [13] was employed for all sequencing reactions (Sequenase; US Biochemical, Cleveland, Ohio, USA). Potential errors arising from *Taq* polymerase were eliminated by directly sequencing three independent clones for each strain using custom oligonucleotide primers. Alignments of the DNA sequences with reference strains (HIV_{Z2Z6}, HIV-2_{ROD}) were performed using the Lipman/Pearson algorithm (IBI Pustell Sequence Analysis Software, New Haven, Connecticut, USA).

Table 2. HIV-1 and HIV-2 infections characterized by various serological and polymerase chain reaction (PCR) techniques.

	HIV-1					HIV-2				
				PCR					PCR	
Patient	VEIA	PEIA	WB	*gag*	*prt*	VEIA	PEIA	WB	LTR	*prt*
Dual reactive										
89867	+	+	+	+	+	+	+	+	+	+
90011	+	+	+	+	+	+	+	+	±	+
Seropositive										
89920	+	+	+	+	+	+	−	±	−	−
90097	+	+	+	+	+	+	−	±	−	−
90151	+	+	+	+	+	+	−	±	−	−
90045	−	−	−	−	−	+	+	+	+	+
Seronegative										
89943	−	−	−	−	−	−	−	−	−	−
89945	−	−	−	−	−	−	−	−	−	−
90156	−	−	−	−	−	−	−	−	−	−
Reference										
CDC855	+	+	+	+	+	−	−	−	−	−
CDC618	−	−	−	−	−	+	+	+	+	+

VEIA, virus lysate-based enzyme immunoassay; PEIA, peptide enzyme immunoassay; WB, Western blot; *prt*, protease gene; LTR, long terminal repeat; ±, indeterminate; CDC, Centers for Disease Control.

Results

Serological analysis

The initial survey study population consisted of patients from 14 non-surgical, non-obstetric medical wards within university teaching hospitals to which individuals with AIDS were frequently referred. Evaluation of the sera from 495 hospitalized patients indicated the presence of HIV-1, HIV-2, and potential HIV mixed infections within the Brazilian population. Whole-virus lysate-based VEIA and WB analysis indicated that 19% (92 out of 495) of all samples were HIV-1-positive alone, 0.4% (two out of 495) were HIV-2-positive alone, and 4% (22 out of 495) were dually reactive. However, subsequent type-specific PEIA analysis revealed that only four sera were reactive to type-specific peptides derived from the immunodominant domain of the HIV-2 transmembrane protein. Two of these samples (numbers 89867 and 90011) were also reactive with HIV-1-specific peptides and were subsequently classified as 'dually reactive'. One of the four HIV-2-seropositive patients was

(a) (b)

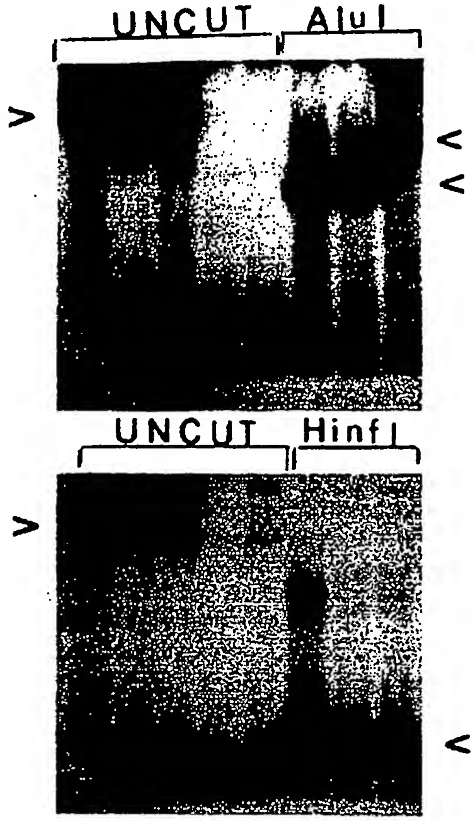

Fig. 1. Identification of polymerase chain reaction (PCR) amplified HIV-1 (a) and HIV-2 (b) protease by oligomer hybridization (OH) technique in two Brazilian patients showing dual HIV-1 and HIV-2 seropositivity. Amplified product was hybridized with a type-specific probe before or after digestion with restriction enzymes: *AluI* for HIV-1 and *HinfI* for HIV-2. →, position of uncut protease gene or its restriction fragments; lane 1, positive controls, pZ6Neo and pMOROD; lanes 2 and 3, samples 89867 and 90011, respectively; 4, negative control (uninfected peripheral blood mononuclear cells); lane 5a, HIV-2 DNA (pMOROD) used as a template for amplification with HIV-1 protease primers and hybridized with HIV-1 probe; lane 5b, HIV-1 DNA (pZ6Neo) used as a template for amplification with HIV-2 protease primers and hybridized with HIV-2 probe.

lost to follow-up. PBMC from the remaining three patients were paired with those from three HIV-1-seropositive patients and three seronegative patients for subsequent analysis of virologic markers. A summary of the serology results of these patients is shown in Table 2.

PCR analysis of the LTR, gag, and protease gene regions

The finding that two Brazilian specimens (numbers 89867 and 90011) exhibited dual seropositivity for both HIV-1 and HIV-2 necessitated additional characterization of their proviral DNA for HIV-1- and HIV-2-specific sequences. To define genetically the infecting HIV serotypes, we screened the patients' PBMC, previously frozen in dimethylsulfoxide, and matched cocultivated specimens by PCR amplification followed by endonuclease cleavage analysis. All samples were analyzed using type-specific primers and probes representative of the HIV LTR, *gag*, and *prt* genes (Table 1). Our findings from direct PCR analysis of the uncultured patients' PBMC were confirmed by subsequent analysis of the matched cocultures. These results are summarized in Table 2. Both HIV-1 *gag*- and HIV-2 LTR-specific sequences were detected in samples from each of the two dual seropositive patients. The extent of amplification observed using the LTR-specific primers was significantly less in patient 90011 than in patient 89867. HIV-1- or HIV-2-specific sequences alone were found selectively in the PBMC of other Brazilian patients and in HIV-positive controls. In contrast, no viral DNA was found in patients with negative serology.

PCR amplification using type-specific primers spanning the HIV-1 or HIV-2 protease genes produced a 300 base pair sequence corresponding to the structural gene (Fig. 1). An HIV-1-specific diagnostic fragment was generated by selective cleavage with *AluI* and hybridization with the type-specific radiolabeled oligonucleotide probe DP18. This fragment was observed in the PCR products from samples of both potential mixed infections. The amplified sequence from sample 90011 resulted in an altered electrophoretic migration pattern because it lacked one of the expected *AluI* cleavages sites. Similarly, an HIV-2-specific diagnostic fragment was produced by cleavage with *HinfI* and detection with the corresponding type-specific probe DP28 (Fig. 1). Such a fragment was again observed in samples from both Brazilian patients with dual seropositivity. End-point dilution analysis of our cloned protease genes indicated that PCR amplification permitted the detection of <10 proviral copies of both HIV-1 and HIV-2 per 0.15×10^6 PBMC. The direct amplification signals observed using HIV-1-specific primers were more intense than the HIV-2-specific products, suggesting a higher HIV-1 proviral copy number within the uncultured PBMC of both specimens 89867 and 90011. These findings not only confirmed those observed for the *gag* and LTR regions, but indicated that specimens 89867 and 90011 each carried distinct subpopulations of HIV-1 and HIV-2 proviral DNA.

Endonuclease analysis of the PCR amplified protease gene

The HIV *prt* gene consists of both conserved and variable regions that are significantly different between HIV-1 and HIV-2 [14]. The endonuclease cleavage patterns of the amplified protease sequences from samples 89867 and 90011 were analyzed to determine if they represented both HIV-1 and HIV-2 or a recombinant virus population within the patients. These results are summarized in Table 3. Following HIV-1-specific amplification, the electrophoretic patterns of the

Table 3. Summary of restriction enzyme digestion patterns analysis of polymerase chain reaction (PCR) amplified HIV-1 and HIV-2 protease gene in two Brazilian samples (numbers 89867 and 90011) and in cloned reference strains.

Protease gene source	Restriction enzymes								
HIV-1	*Alu*I*	*Asu*I	*Ava*II	*Bst*XI	*Bcl*I	*Eco*RII	*Eco*RV	*Mbo*I	*Ssp*I
Patient									
89867	+2	+	+	+	+	+	+	+	–
90011	+1	+	+	+	–	+	–	–	–
Reference									
pMO855	+2	+	+	+	+	+	–	+	–
pZ6Neo	+3	+	+	+	+	+	–	+	–
HIV-2	*Asu*I	*Bst*XI	*Dde*I	*Hin*fI	*Hae*III	*Rsa*I	*Ssp*I		
Patient									
89867	–	–	+	+	–	+	+		
90011	–	–	+	+	–	+	+		
Reference									
pMOROD	+	–	+	+	+	+	+		
pMO618	–	–	+	+	+	+	+		

*(n) variable number of cleavage sites present; †, identical cleavage pattern; –, no cleavage sites.

*Asu*I, *Ava*II, *Bst*XI, and *Eco*RII cleavage products were similar to our HIV-1 laboratory reference strains. Digestion patterns for *Alu*I, *Bcl*I, *Eco*RV, and *Mbo*I differed from those of the HIV-1 reference strains. In sample 90011 one of two restriction sites for *Alu*I was missing and no digestion was observed with *Bcl*I or *Mbo*I. *Eco*RV digested one of two Brazilian samples while the laboratory strains pMO855 and pZ6Neo lacked this cleavage site. Restriction analysis of the amplified HIV-2 protease sequences was consistent with the HIV-2 prototype gene as seen in the *Dde*I, *Hin*fI, *Rsa*I, and *Ssp*I restriction digests. The cleavage patterns observed with *Asu*I or *Hae*III distinguished the Brazilian samples from the HIV-2 reference strains. In addition, the *Asu*I restriction patterns of the amplified HIV-2 protease genes also differed from those of the amplified HIV-1 genes. The HIV-1-specific *Bst*XI cleavage was absent following HIV-2-specific amplification in all samples, and conversely the HIV-2-specific *Ssp*I cleavage site was absent in all samples amplified from HIV-1-specific primers.

Sequence of PCR-amplified HIV protease gene

Finally, we completed the above identification by cloning and sequencing PCR-amplified protease genes from both HIV-1 and HIV-2 in the two Brazilian samples. Alignments of the amplified protease sequences from these samples with HIV-1 (HIV_{Z2Z6}) and HIV-2 (HIV-2_{ROD}) reference strains are presented in Fig. 2. The Brazilian HIV-1 and HIV-2 protease gene sequences retained 95 and 93% homology with their prototype strains, respectively. Within a given serotype most of the observed variations were synonymous point mutations. Between HIV-1 and HIV-2 serotypes the nucleic acid sequences differed by 40%. At the amino-acid level, the sequences differed by as much as 64% between HIV-1 and HIV-2 isolates.

Discussion

The initial serologic indicators within this collaborative study showed a seroprevalence of approximately 1.7% among reactive patients for HIV-2, while 19% of the reactive sera responded with both HIV serotypes. The type-specific serological and genetic analysis presented here shows that 1.7% of seropositive patients were infected with HIV-2 with only an additional 1.7% infected with both HIV-1 and HIV-2. PCR detection of HIV proviral sequences from the LTR, *gag*, and *prt* gene regions confirmed the presence of HIV-1 and HIV-2 mixed infections in two Brazilian patients who were seropositive for both viruses. The proviral sequences amplified from these patients, using type-specific protease gene primers, were similar to the homologous prototype HIV gene but distinct from those of reference strains. This indicated that they did not arise from laboratory contamination of the specimens. These findings are consistent with the selective amplification of distinct HIV-1 and HIV-2 isolates from the mixed infections. They further demonstrate that the architecture of the protease gene region in each is comparable to that of the prototype strains and inconsistent with a new or variant virus type. Our data emphasize the validity of PCR analysis of viral protease gene for the identification of mixed HIV infections. Using this protocol it was possible to confirm the presence of both HIV-1 and HIV-2 DNA in the same person using three different methodologies; oligomer hybridization, restriction enzyme digestion and analysis of restriction patterns, and DNA sequencing.

HIV-2 infection is rare outside West Africa and less is known concerning its pathogenesis than for HIV-1, the principal etiologic agent of the current AIDS pandemic. In regions with a low prevalence of HIV-2, such as Brazil, the likelihood that an individual would un-

```
(a)

HIVZ2Z6   CCTCAAATCACTCTTTGGCAACGACCCCTTGTTACAATAAAAATAGGGGG  50
89867     .............................C..C........G........
90011     .............................C..C................A

HIVZ2Z6   ACAGCTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAG 100
89867     G..A..............................................
90011     G.................................................

HIVZ2Z6   AAGAAATGAATTTGCCAGGAAAATGGAAACCAAAAATGATAGGGGGAATT 150
89867     ...G.................G............................
90011     ....C...C.........................................

HIVZ2Z6   GGAGGTTTTATCAAAGTAAGACAGTATGATCAAATACTCATAGAAATCTG 200
89867     ................................G...TC............
90011     ...................A.........G..G....C............

HIVZ2Z6   TGGGCATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACA 250
89967     ...A..G....................GA.....................
90011     C..A..T...........................................

HIVZ2Z6   TAATTGGAAGAAATTTGTTGACCCAGATTGGCTGCACTTTAAATTTT    297
89867     ..............C.......T........................
90011     ..............C C.....T........................

(b)

HIV2ROD   CCTCAATTCTCTCTTTGGAAAAGACCAGTAGTCACAGCATACATTGAGGG  50
89867     ............................................C.....
90011     ...........................A................C.....

HIV2ROD   TCAGCCAGTAGAAGTCTTGTTAGACACAGGGGCTGACGACTCAATAGTAG 100
89867     G.....G.........C.A..G..............T.............
90011     A.....G.........C.A..G..............T.............

HIV2ROD   CAGGAATAGAGTTAGGGAACAATTATAGCCCAAAAATAGTAGGGGGAATA 150
89867     .................G.......C.CT......G....G.........
90011     ...A.............G.......C.CT...........G.........

HIV2ROD   GGGGGATTCATAAATACCAAGGAATATAAAAATGTAGAAATAGAAGTTCT 200
89867     ...............C....A.....................A....A..
90011     ...............C....A.....................A....A..

HIV2ROD   AAATAAAAAGGTACGGGCCACCATAATGACAGGCGACACCCCAATCAACA 250
89867     ........GA...A.A.................A................
90011     ........GA...A.A..................................

HIV2ROD   TTTTTGGCAGAAATATTCTGACAGCCTTAGGCATGTCATTAAATCTA    297
89867     ...............................................
90011     ...............................................
```

Fig. 2 Comparison of protease gene sequences. (a) HIV-1; (b) HIV-2; (-------), sequence identity with reference strain.

dergo multiple independent heterotypic HIV infections is small; consequently, recognition of a single such individual would be rare. Common risk factors have been associated with the transmission of both HIV serotypes. Individuals repeatedly involved in high-risk activities form a cluster of individuals predisposed to the entry and expansion of a new variant within the population. Further, should there by synergistic effects in the pathogenesis of multiple HIV infections, these individuals would be among the first to present clinical manifestations of disease. Broad seropositivity has been associated with increased clinical manifestations, and mixed infections have only been documented in hospitalized patients to date [8,15].

The prevalence of mixed HIV infections in Brazil cannot be accurately determined from this small study; it will require analysis of a much larger number of HIV-infected individuals in Brazil. However, it is clear that there are currently both HIV-2 only and mixed HIV-1- and HIV-2-infected individuals in Brazil.

While most individuals possessing antibodies to HIV-1 and HIV-2 have not been dually infected, we have identified two mixed HIV-infections and one HIV-2-infection in our selective study of hospitalized Brazilian patients. Sample 89867 was from a 31-year-old homosexual man with culture-proven pulmonary tuberculosis, oral candidiasis, suspected systemic and cerebral toxoplasmosis, dematophytosis, weight loss >10%, and hepatosplenomegaly. He was born in Alagoas, Brazil, but resided in the state of Rio de Janeiro. Sample 90011 was from a 25-year-old homosexual man with disseminated tuberculosis and pleural effusion, oral candidiasis, anal herpes simplex, jaundice, weight loss >10%, diarrhea, and hepatosplenomegaly. Both his birthplace and residence were Rio de Janeiro. Patient 90045, who was HIV-2-infected only, was a 48-year-old woman who had received a blood transfusion, and was admitted with cough of >1 month's duration, bloody sputum, fever, and shortness of breath. Chart review noted findings of bronchiectasis, oral candidiasis, papular dermatitis, and gastrointestinal strongyloidiasis. To date, we have been unable to determine if either of these individuals has travelled to West Africa or has had contact with individuals who have done so. Although it remains unclear whether infection with a given strain of HIV will inhibit subsequent infections with homotypic strains, the demonstration of naturally occurring mixed HIV infections implies that infection does not preclude subsequent superinfection with quite distinct HIV strains. Similarly, mixed HIV infections may be readily established *in vitro* [16].

This study demonstrates the utility of genetic techniques in characterizing potential mixed infections from regions with greatly dissimilar prevalences for HIV-1 and HIV-2. The findings also serve to emphasize the need to analyze virologic, as well as serologic, markers to gain a better insight into the relative proportions of virus types circulating within a community. Finally, our data support the conclusion that predictions based entirely on serology may overinterpret the prevalence of mixed HIV infections.

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The One-tube Quantitative HIV-1 RNA NASBA: Precision, Accuracy, and Application

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Over the past few years the quantitation of nucleic acids with target sequence amplification methods has been accomplished in many ways. Theoretically the coamplification of internal standard nucleic acid sequences, either DNA or RNA, with the wild-type nucleic acid is superior to other quantitation methods, provided that the amplification efficiencies of wild-type nucleic acid and internal standard nucleic acid are equal.(1,2) This method has been applied successfully for PCR,(3), RT–PCR,(4,5) and NASBA.(6)

NASBA is an isothermal nucleic acid amplification method that, like 3SR,(7,8) evolved from TAS,(9) the first RNA transcription-based amplification method described. Nucleic acid amplification in NASBA is accomplished by the concerted enzyme activities of AMV reverse transcriptase, RNase H, and T7 RNA polymerase, resulting in the accumulation of mainly single-stranded RNA that can readily be used for detection by hybridization methods. The application of an internal RNA standard to NASBA resulted in a quantitative nucleic acid detection method with a dynamic range of four logs but which needed six amplification reactions per quantitation.(6) Recently, this method was improved dramatically by the application of multiple, distinguishable, internal RNA standards added in different amounts and the electrochemiluminesence (ECL) detection technology.(10) This one-tube quantitative (Q)-NASBA uses only one amplification per quantitation and enables the addition of the internal standards to the clinical sample in a lysis buffer prior to the actual isolation of the nucleic acid.(10) This approach has the advantage that nucleic acid isolation efficiency has no influence on the outcome of the quantitation, which is in contrast to methods in which the internal standards are mixed with the wild-type nucleic acid after its isolation from the clinical sample.

The specific amplification of single-stranded RNA in the presence of double-stranded DNA because of the isothermal nature of NASBA has led to clinical applications in which detection of RNA is essential, such as HIV-1 RNA in viral particles,(6,10,11) rRNA in Mycobacteria,(12,13) chronic myeloid leukemia (CML) mRNA in blood lymphocytes,(14) and hepatitis C virus, (HCV) RNA in viral particles.(15) The first application of the one-tube Q-NASBA was developed for the quantitation of HIV-1 RNA. However, for clinical diagnostic applications of the one-tube HIV-1 Q-NASBA one should have a good assessment of the precision and the accuracy of the method in the dynamic range that is covered by the internal standards. Only then can firm conclusions concerning changes in HIV-1 viral RNA load, that are not attributable to variation in the quantitative assay itself, be made.

In this study we describe the precision and accuracy of the one-tube HIV-1 Q-NASBA. In this respect, precision is defined as the variation (standard deviation) between quantitative results obtained from the same sample and accuracy is defined as the difference between the quantitative result and the actual input.

This latter objective can be accomplished by the use of a "gold standard" for which an in vitro cultured HIV-1 viral stock solution was obtained. In this stock solution the amount of viral particles was determined using electron microscopy.(11,16,17) Another important application of the gold standard lies in the comparison of quantitative HIV-1 RNA results obtained using different methods at different laboratories. As more and more quantitative HIV-1 RNA detection methods emerge, the widespread use of a gold standard can help to resolve the discrepancies found in viral RNA load at different laboratories.(17)

MATERIALS AND METHODS

All one-tube HIV-1 Q-NASBA assays were performed using the NASBA HIV-1 RNA QT kit from Organon Teknika (Turnhout, Belgium), containing all nec-

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essary reagents for the nucleic acid isolation, amplification, and detection as described below. A flowchart describing the procedure is shown in Figure 1.

Nucleic acids were extracted from HIV-1 viral particles added to 100 μl of plasma and 900 μl of guanidine thiocyanate (GuSCN) lysis buffer. The amount of HIV-1 viral particles was determined using electron microscopy.[16] Before extraction, three RNA internal standards (Q_A, Q_B, and Q_C) from the NASBA HIV-1 RNA QT kit were added to this lysis mixture. The Q_A, Q_B, and Q_C internal standard RNAs only differ from the wild-type RNA and each other by a 20-nucleotide randomized sequence with the same nucleotide composition. This design of internal standards ensures equal efficiency of isolation and amplification. Subsequently 50 μl of activated silica particle solution was added, binding all nucleic acids (DNA and RNA), in the lysate.[18] The silica particles were washed with GuSCN wash buffer, 70% ethanol, and acetone. After drying, the silica particles, the nucleic acids were eluted in 50 μl of elution buffer (1 mM Tris at pH 8.5).

Five microliters of the extracted nucleic acids was used as the input for amplification by the addition of 5 μl of primer mix, followed by a 5 min incubation at 65°C. The reaction was incubated at 41°C, for 5 min, followed by the addition of 5 μl of enzyme mix and further incubation for 90 min at 41°C. The amount of Q_A, Q_B, Q_C and wild-type amplified products were determined using the ECL detection method[10] in the NASBA QR SYSTEM instrument.

Five microliters of 20-fold diluted amplified products was added to four tubes. Subsequently, 10 μl of magnetic bead solution with a generic probe for binding all amplified products, 10 μl of probe solution, specific for Q_A, Q_B, Q_C, and wild type sequences respectively, were added to each tube. Hybridization was carried out at 41°C for 30 min. The tubes were then placed in the NASBA QR SYSTEM ECL detection instrument, and 300 μl of assay buffer (0.1 M TPA) was added.

The magnetic beads carrying the hybridized products/probe complex are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the ECL reaction. The light emitted by the hybridized ruthenium-labeled probes is proportional to the amount of product. Calculations based on the relative amounts of the four products reveal the original amount of wild-type HIV-1 RNA in the sample.

In our laboratory, we have applied physical separation of our NASBA activities (nucleic acid isolation, amplification setup, and detection of amplified products) to prevent carryover contamination. Physical separation was accomplished by the use of different laboratories or the use of fume hoods and flow cabinets. Every designated area is supplied with its own set of laboratory equipment (pipettes), and movement between areas is minimized.

During this study we analyzed 35 negative controls that were placed among positive samples over a period of weeks. Only once did we observe a false-positive result which, upon retesting of the amplified nucleic acid, became negative, indicating a carryover contamination event during the detection setup. During this study we have not observed any cross contamination during nucleic acid isolation or amplification setup.

DYNAMIC RANGE

The dynamic range of the one-tube Q-NASBA is defined by the concentrations of the internal standards (i.e., Q_A, Q_B, and Q_C RNA). Previously,[10] we have shown that accurate quantitation is possible between a factor 10 below the lowest internal standard (Q_C) and a factor 10 above the highest internal standard (Q_A). The amounts used in this study are 10^6, 10^5, and 10^4 RNA molecules of Q_A, Q_B, and Q_C, respectively. This enables quantitation of the

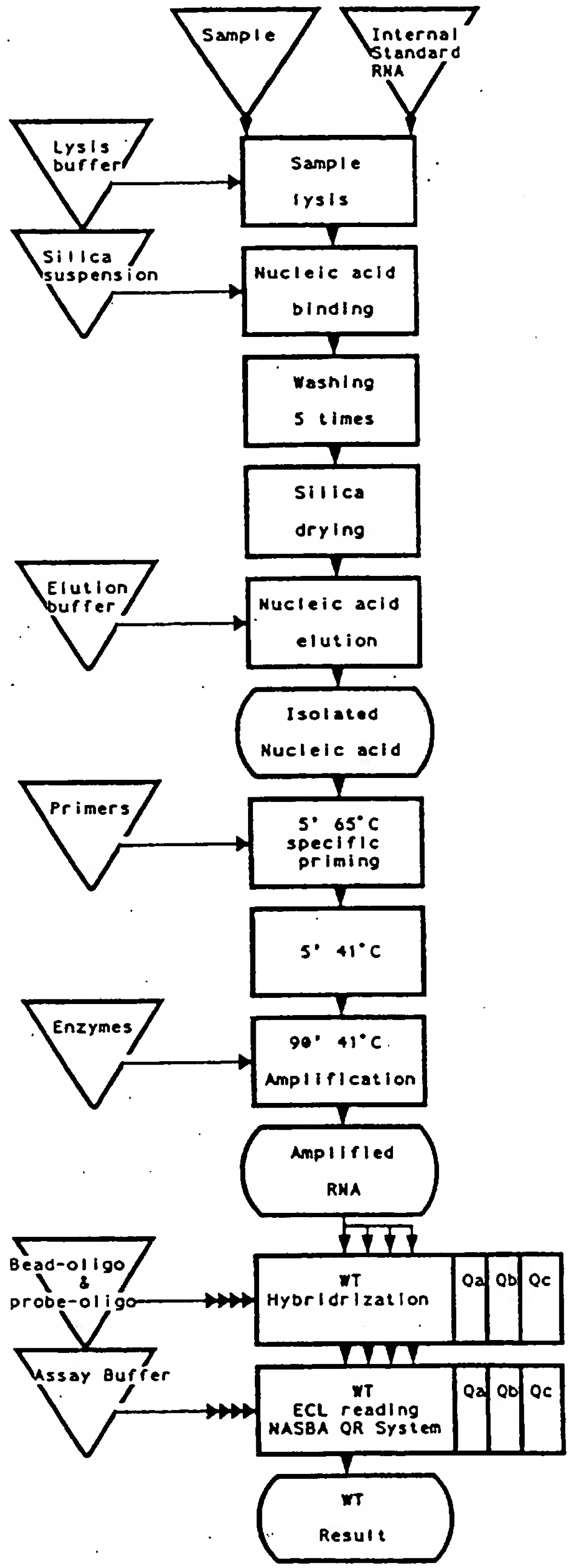

FIGURE 1 Flowchart of the experimental procedure of the HIV-1 RNA NASBA QT kit.

wild-type HIV-1 RNA between 10^3 and 10^7 molecules. The dynamic range of the one-tube Q-NASBA was determined using a dilution series of HIV-1 virus in plasma (100-µl aliquots), containing 1.86, 2.86, 3.86, 4.86, 5.86, and 6.56 log wild-type RNA molecules, respectively. As shown in Figure 2 the quantitation of the lowest wild-type HIV-1 RNA input level (i.e., 1.86 log) revealed an inaccurate result that was not used for linear regression of the data points. The lowest wild-type RNA input level that was quantitated accurately in this study was 2.86 log (725 RNA molecules). In other studies wild-type RNA input levels of 2.6 log (400 RNA molecules) could be quantitated accurately (data not shown).

PRECISION AND ACCURACY

For clinical diagnostic application of quantitative HIV-1 RNA assays it is important to know the precision and accuracy of the assay. Only then can one discriminate between fluctuations in HIV-1 viral load results that are attributable to variation in the assay or to differences in HIV-1 viral load as a consequence of factors such as disease stage or antiviral therapy. In this matter, precision is defined as fluctuations in quantitative results obtained on the same amount of HIV-1 RNA input (i.e., the standard deviation). The accuracy is defined as the difference between the outcome of the quantitation and the actual input of HIV-1 RNA. To determine both, we used three concentrations of the HIV-1 virus stock solution (i.e., the gold standard) as input in 100 µl of plasma and 900 µl of lysis buffer and quantitated each concentration 20 times. The results are summarized in Table 1.

The precision was found to increase with increasing wild-type HIV-1 RNA input concentration; precision of 0.23 log was observed when quantitating 3.34 log wild-type HIV-1 RNA input, whereas the quantitation of 6.34 log wild-type HIV-1 RNA input was accomplished with a precision of 0.13 log. These results are in good agreement with the results we obtained previously.[10] The accuracy of the one-tube Q-NASBA ranges from 0.02 to 0.19 log, indicating the correct calibration of the internal standards (Q_A, Q_B, and Q_C RNA) used in the assay.

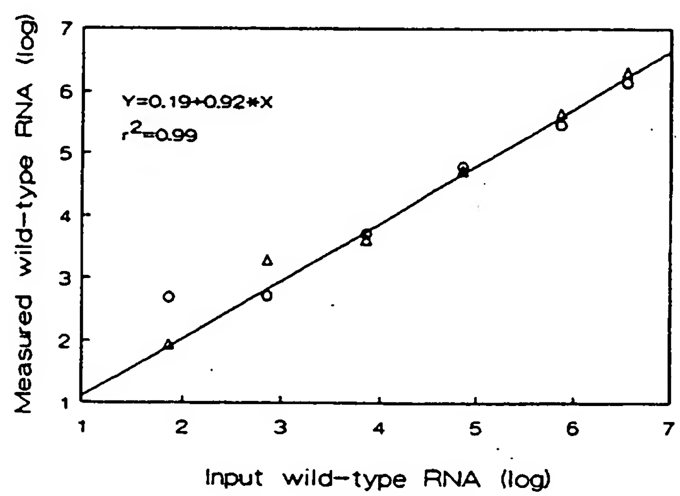

FIGURE 2 Dynamic range of the one-tube Q-NASBA using 10^6, 10^5, and 10^4 RNA molecules of Q_A, Q_B, and Q_C, respectively. The internal standards were mixed with 1.86, 2.86, 3.86, 4.86, 5.86 and 6.56 log wild-type HIV-1 RNA molecules (i.e., dilution series of HIV-1 virus stock solution) in 100 µl of plasma and 900 µl of lysis buffer. All quantitations were performed in duplicate. For curve fitting (i.e., linear regression), the wild-type RNA quantitation of 1.86 log was not taken into account.

TABLE 1 Precision and Accuracy of the One-tube Q-NASBA

	Input wild-type HIV-1 RNA molecules (log)		
	3.34	4.86	6.34
Number	19[a]	20	20
Mean	3.23	4.84	6.15
Precision (= std)	0.23	0.22	0.13
Accuracy	0.11	0.02	0.19

[a]One data point was found to be an outlier by Grubbs' test.

Using a standard deviation of 0.23 (Table 1) for the one-tube Q-NASBA, differences found in HIV-1 viral load that are ≥1 log are caused by external factors, like disease stage or antiviral therapy, other than fluctuations in the one-tube Q-NASBA ($P=0.0035$). Differences in the HIV-1 viral load of 0.5 log found as a result of single measurements can be subscribed to external factors with $P=0.13$. However, when differences of 0.5 log are found as a result of duplicate measurements, these can be described to external factors with more certainty ($P=0.03$).

INFLUENCING FACTORS

The one-tube Q-NASBA assay results might be influenced by external factors related to the clinical sample used for analysis. For this reason, the gold standard (input 4.85 log wild-type HIV-1 RNA) was quantitated with addition of 100 μl of citrate, heparin, or EDTA plasma, or serum. The results are shown in Figure 3. There appears to be no difference in the quantitation of the gold standard in these four sample types. However, the influence of the coagulation process on the quantitation of HIV-1 viral RNA was not investigated in this experiment. Possibly, HIV-1 viral particles are trapped during the coagulation for obtaining serum, resulting in lower viral load results when compared with quantitations performed on plasma samples. To address this problem, comparisons should be made between quantitation of HIV-1-RNA in plasma and serum samples of HIV-1-infected individuals.

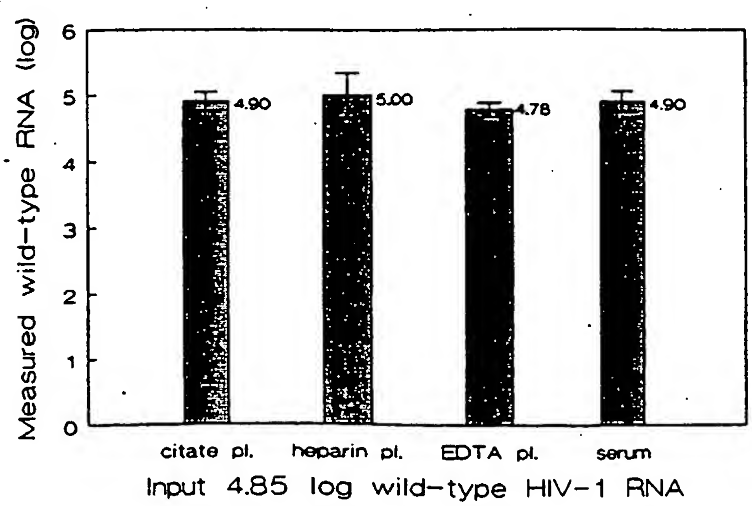

FIGURE 3 Wild-type HIV-1 RNA (4.85 log) was quantitated five times in citrate plasma, heparin plasma, EDTA plasma, and serum, respectively. The mean result of the quantitations is given next to each bar.

Three components of plasma and serum that could possibly influence the quantitative outcome of a one-tube Q-NASBA are lipids, hemoglobin, and albumin. These components were added in increasing concentrations to 100 μl of normal human serum (reference serum) and 900 μl of lysis buffer in which 4.85 log wild-type HIV-1 RNA was spiked. Neither lipid (3–10%), hemoglobin (40–140 μM), nor albumin (50–70 g/liter) influenced the outcome of the quantitation (Fig. 4). During the nucleic acid isolation procedure, these compounds are separated from the nucleic acid in such a way that concentrations remaining in the eluate are too low to influence the NASBA amplification.

APPLICATIONS

The advantages that NASBA has over other amplification methods (PCR, LCR, SDA, etc.) for the amplification of RNA has led primarily to the development of NASBA assays for those applications where RNA is a diagnostic target. The determination of HIV-1 viral RNA load is such an application. Both as a research tool and as a diagnostic assay, one can appreciate the value of the one-tube Q-NASBA. The simplicity of the assay, combined with the technical advantages when compared with other quantitative assays, allows quantitative results to be obtained on a large scale. For HIV-1 it is obvious that viral RNA will be an important marker in the research for vaccines and antiviral drugs. In a recent study, the one-tube Q-NASBA was used to assess the efficacy of AZT therapy in asymptomatic HIV-1-infected individuals (S. Jurriaans, pers. comm.). The results of two representative individuals, a responder, patient 8301, and a nonresponder, patient 8306, are depicted in Figure 5. Differences in HIV-1 viral load ≥1 log cannot be explained by variation in the one-tube Q-NASBA itself and are therefore explained as effects of the AZT therapy. These results illustrate the useful application of this assay for the rapid assessment of antiviral therapy. In this way, the one-tube Q-NASBA can serve as an important tool in the search for new antiviral drugs.

CONCLUSIONS

A quantitative nucleic acid detection assay is generally composed of three components: nucleic acid extraction, amplification, and detection. The qual-

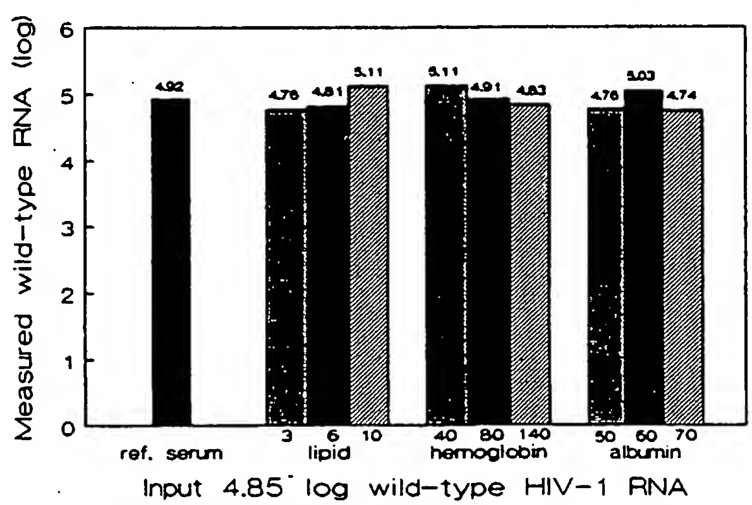

FIGURE 4 Wild-type HIV-1 RNA (4.85 log) was quantitated in normal human serum (reference serum) with the addition of lipid (3%, 6%, and 10%), hemoglobin (40, 80, and 140 μM) or albumin (50, 60, and 70 g/liter). The results of the quantitations are stated next to each bar.

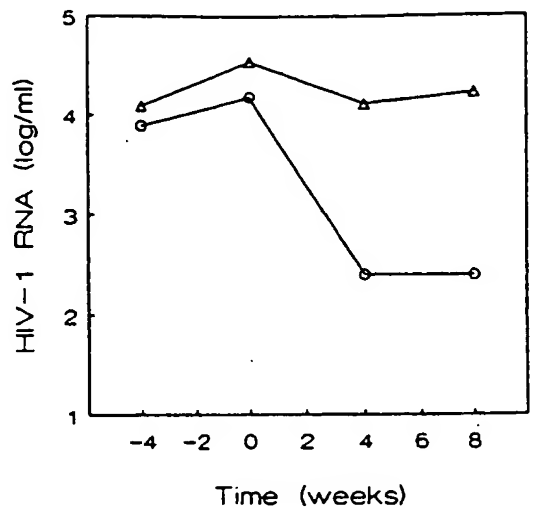

FIGURE 5 HIV-1 RNA load in plasma of two asymptomatic HIV-1-infected individuals treated with AZT. The AZT therapy was started at week 0. (O) patient 8301; (Δ) patient 8306.

ity of the quantitative result is highly dependent on the efficiency and reproducibility of each of the components. In the one-tube Q-NASBA assay the addition of multiple, distinguishable internal standard RNAs to the clinical sample prior to nucleic acid isolation abolishes the effect of nucleic acid isolation efficiency on the quantitation outcome. The ratio of wild-type RNA to internal RNA standards will remain constant independent of the overall extraction efficiency.[10] This ratio determines the quantitative outcome of the assay. Furthermore, because the amplification efficiencies of wild-type and internal standard RNAs are equal, the ratio of these RNAs before amplification will be reflected in the amplification products. This implies that detection of amplified products needs to be absolutely quantitative to determine accurately the ratio of wild-type RNA to internal RNA standards. The ECL detection technology has this property and can measure hybridization signals with a dynamic range of 5 orders of magnitude.[19,20]

The one-tube Q-NASBA consisting of Boom nucleic acid isolation technology,[18] NASBA amplification,[21] and ECL detection[19,20] has a dynamic range of 4×10^2–4×10^7 RNA molecules when using 10^6, 10^5, and 10^4 RNA molecules of the Q_A, Q_B, and Q_C internal standards, respectively (Fig. 1). Within this dynamic range the precision and accuracy of the assay enables measurement of the changes in HIV-1 RNA load of ≥ 1 log and, very probably, changes of ≥ 0.5 log can be subscribed to factors other than variation in the assay. The use of the Boom nucleic acid isolation method[18] makes the assay suitable for almost any kind of clinical sample (plasma, serum, whole blood, cells). Because of the inclusion of efficient washing steps of the nucleic acids bound to silica, possible NASBA inhibiting factors are removed.

As shown in this study, the concern about contamination that exists for all amplification methods can be dealt with simply by the physical separation of the different NASBA activities (see Materials and Methods). Together with the simplicity of the one-tube Q-NASBA assay, this enables the widespread use of this assay for large-scale quantitation of HIV-1 RNA both in research and clinical settings. With the introduction of a gold standard, enabling comparisons of different quantitation methods, the way will be opened for the application of these assays as a widespread tool in vaccine development and antiviral drug research.

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